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Critical Role of the IFN-Stimulated Gene Factor 3 Complex in TLR-Mediated IL-27p28 Gene Expression Revealing a Two-Step Activation Process

Céline Molle, Michel Goldman, and Stanislas Goriely

In myeloid dendritic cells, activation of the IL-27p28 gene is selectively induced by ligands of TLR4 or TLR3, both coupled to the Toll/IL-1R-related domain-containing adaptor-inducing IFN/IFN regulatory factor (IRF)3 pathway. In response to both ligands, autocrine type 1 IFN production was required for optimal IL-27p28 expression. Type I IFN signaling was necessary for sustained IRF1 activation and formation of the IRF9-containing IFN-stimulated gene factor 3 complex. Indeed, we demonstrated that IRF1 and IRF9 are sequentially activated and recruited to the IL-27p28 IFN-stimulated regulatory element site. Involvement of IRF1 and IRF9 in the induction of IL-27p28 was confirmed in vitro and upon in vivo exposure to TLR ligands. Thus, in response to TLR4 or TLR3 ligation, the initial induction of the IL-27p28 gene depends on the recruitment of IRF1 and IRF3, whereas transcriptional amplification requires recruitment of the IFN-stimulated gene factor 3 complex. These results highlight the complex molecular interplay between TLRs and type 1 IFNs for the control of IL-27 synthesis.


Materials and Methods

Mice

LPS2- (27) and IRF1-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IRF3-, IRF7-, and IRF9-deficient mice (21) were obtained from the Riken BioResource Center (Ibaraki, Japan), with the approval of T. Taniguchi (University of Tokyo, Tokyo, Japan). Type 1 IFN receptor (IFNAR)-deficient mice were kindly provided by Dr. G. Warnier (Ludwig Institute, Brussels, Belgium). Mice were bred and maintained in specific pathogen-free conditions according to institutional guidelines.

Cells and reagents

Murine bone-marrow derived dendritic cells (DCs) were generated as previously described (28). The HEK-293 human kidney cell line was obtained from LGC Promochem (Tedddington, U.K.). Ultrapure LPS from Escherichia coli (0111:B4) was obtained from Cayla (Toulouse, France). Polyinosine-polycytidylic acid [poly(I:C)] was purchased from GE Healthcare (Ghent, Belgium). Recombinant murine IFN-β was obtained from PBL Interferon Source (Piscataway, NJ). For LPS and poly(I:C) challenge, each mouse was injected i.p. with 10 and 5 mg/kg, respectively.

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Plasmid constructs
The plasmid p28lacWT and p28lac MutAB were described previously (23). IRF3 5D, IRF1, and IRF7 4D expression vectors were kindly provided by R. Lin (McGill University, Montreal, Quebec, Canada). The IRF9 Stat2 expression vector was provided by C. Horvath (Evaston Northwestern Healthcare Research Institute, Evanston, IL).

Quantification of cytokine production in sera and culture supernatants
Murine IL-27p28 levels were determined in cell-free supernatants or serum by a specific ELISA kit (Quantikine; R&D Systems, Abingdon, U.K.) with detection limits of 3.9 pg/ml; murine IFN-β levels were determined in supernatants using an ELISA kit (PBL Interferon Source) with detection limits of 15.6 U/ml. Murine IL-12p70 and -23 levels were determined in serum using DuoSet ELISA (R&D Systems) with detection limits of 15 pg/ml and Ready-SET Go ELISA (E Bioscience, San Diego, CA) with detection limits of 30 pg/ml, respectively.

RNA purification and real-time RT-PCR
Total RNA was extracted using a MagnaPure LC RNA-High Performance Isolation Kit (Roche Diagnostics, Brussels, Belgium). Reverse transcriptase and real-time PCR reactions were carried out using LightCycler 480 RNA Master Hydrolysis Plus probes (one-step procedure) or a LightCycler 480 Real-Time PCR system (Roche Diagnostics). Primer sequences are described in Supplemental Table 1.

Immunoblotting
Bone marrow DCs were harvested, washed twice with cold PBS, and directly lysed in cell lysis reagent (10 mM Tris-HCl, 150 mM NaCl, 1.5 mM MgCl2, and 1% Igepal). Protein concentration was evaluated by Bradford assay (Bio-Rad, Hercules, CA). An equal amount of proteins was resolved by 8% SDS-PAGE and immunoblotted. Membranes were then probed with the following Abs: anti-IRF1 (sc-640), anti-Stat2 (sc-950), anti-IRF3 (51-3200) were obtained from R&D Systems and Invitrogen, Stat2 Abs were obtained from Santa Cruz Biotechnology; anti-IRF9 (AF5629) was provided by R. Lin (McGill University, Montreal, Quebec, Canada). The IRF9 Stat2 binding-reaction mixture. Purified IRF7-GST protein was kindly provided by a specific ELISA kit (Quantikine; R&D Systems, Abingdon, U.K.) with detection limits of 30 pg/ml, respectively.

Immunofluorescence
DCs were adhered onto glass coverslips for ≥1 h. Cells were fixed with PBS containing 4% paraformaldehyde. For the staining, cells were permeabized with PBS containing 0.1% Triton X-100. Cells were then incubated with PBS BSA 10%. This incubation was followed by application of the following primary Abs: anti-IRF1 (sc-640), anti-Stat2 (sc-950), anti-IRF3 (sc-950), anti-IRF7 (sc-2961) anti-vimentin (sc-32322) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IRF9 (AF5629, R&D Systems), and anti-P-IRF3 (4947, Cell Signaling Technology, Beverly, MA). Immunoreactive bands were revealed using the ECL advanced detection method (GE Healthcare).

EMSAs
EMSAs were performed as previously described (28). For supershift assays, polyclonal Abs against IRF3 (51-3200, Invitrogen, Cambridge CA), IRF1 (sc-640), or Stat2 (sc-950) (Santa Cruz Biotechnology) were added to the binding-reaction mixture. Purified IRF7-GST protein was kindly provided by R. Lin (McGill University, Montreal, Quebec, Canada).

Transient transfection and luciferase assays
HEK 293 cells were transfected using FuGENE-6 (Roche Diagnostics), as previously described (28). Promoter activities were analyzed using the Dual-Glo Luciferase Reporter Assay system (Promega, Madison, WI). Promoter activities were then normalized to Renilla luciferase activities.

Chromatin immunoprecipitation assay
The chromatin immunoprecipitation (ChIP) experiments were performed using the Magna ChIP G Chromatin Immunoprecipitation Kit from Millipore (Billerica, MA) according to the manufacturer’s instructions. Anti-IRF1 and anti-Stat2 Abs were obtained from Santa Cruz Biotechnology; anti-IRF9 (AF5629) and anti-IRF3 (51-3200) were obtained from R&D Systems and Invitrogen, respectively. Primer sequences are detailed in Supplemental Table 1.

Splenic CD11c+ DC isolation
Splenocytes were dissociated with a 400 U/ml type IV collagenase solution (CLSIII, Worthington Biochemical, Lakewood, NJ) in HBSS for 20 min at 37˚C. Organs were teased apart, and the resulting mixture was passed through a nylon filter. Cells in suspension were centrifuged in HBSS-EDTA (10 mmol) and separated by density centrifugation using a Nycocon gradient (density = 1.085 g/ml, Nycomed, Oslo, Norway) at 1700 × g for 15 min at 4˚C. The interface cell fraction was collected, and CD11c+ cells were purified by incubation with anti-mouse CD11c (N418) microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) in 100 µl PBS supplemented with 5 mmol EDTA and 1% FCS for 20 min at 4˚C. CD11c+ DCs were enriched to >95% purity by positive selection over a MACS separation column (Miltenyi Biotec).

Statistical analysis
According to the Gaussian distribution of the values, the Student t test with Welch correction or the Mann–Whitney U test was used for statistical analysis.

Results
Differential implication of IRF3 in LPS- and poly(I:C)-mediated IL-27p28 production
We previously showed that LPS and poly(I:C) were efficient inducers of IL-27p28 by DCs. We also provided evidence that the TRIF/IRF3 pathway plays a critical role in the expression of the p28 subunit induced by LPS (23). We further investigated the involvement of this pathway in poly(I:C) stimulation. Under these conditions, IL-27 production also required activation of the TRIF pathway (Supplemental Fig. 1). In contrast to LPS stimulation, IRF3−/− DCs were able to produce substantial levels of IL-27 when exposed to poly(I:C), although at lower levels than wild-type (WT) cells (Fig. 1A). We then quantified mRNA levels for the p28 subunit at different time points after stimulation. Compared with LPS treatment, the kinetics of p28 mRNA production were delayed and sustained in response to poly(I:C) (Fig. 1C, 1D). In the absence of IRF3, induction of IL-27p28 by poly(I:C) was decreased at early time points (2–6 h poststimulation) but was maintained at later time points (9–12 h poststimulation) (Fig. 1D). Because IRF3 is directly implicated in the induction of type I IFNs, we investigated IFN-β production in the different groups. Consistent with previous reports, we found that production of IFN-β in response to LPS was largely dependent on IRF3 (29). In contrast, in response to poly(I:C), the production of IFN-β was still detectable in IRF3−/− DCs (Fig. 1B).

Type I IFN signaling is required for IL-27p28 expression induced by TLR ligation
Based on these results, we hypothesized that upon poly(I:C) stimulation, the residual production of type I IFN by IRF3−/− DCs could contribute to IL-27 gene expression in an autocrine manner. To test this possibility, we used DCs derived from IFNAR-deficient mice. In the absence of type I IFN signaling, the production of IL-27 was greatly decreased upon LPS stimulation and completely abrogated upon poly(I:C) stimulation (Fig. 1E). Initial IL-27p28 gene activation (2 h poststimulation) was preserved in LPS-stimulated IFNAR−/− DCs, but mRNA levels rapidly decreased thereafter (Fig. 1F). In comparison, poly(I:C)-mediated IL-27p28 gene activation was completely dependent on type I IFN signaling, even at early time points (Fig. 1G). These results indicate that the autocrine feedback loop initiated by type I IFN is critical for efficient IL-27p28 expression in response to LPS and poly(I:C). However, it is dispensable for the early induction of p28 transcriptional activity in response to LPS.

Autocrine type I IFNs signal through IRF1 and IRF9 upon LPS or poly(I:C) stimulation
To understand the molecular mechanisms responsible for these observations, we analyzed the events initiated by type I IFN signaling in the context of LPS or poly(I:C) stimulation. The binding
of IFN-α/β to the type I IFN receptor leads to the formation of two transcriptional activator complexes: signal transducer and activator of transcription (Stat) 1 homodimers and a Stat1/Stat2/IRF9 heterodimeric complex known as ISGF3. Stat1 dimers also induce the IRF1 gene by its binding to the IFN-γ activation site, whereas ISGF3 activates IFN-inducible genes, including IRF7, through an ISRE binding site (30).

First we assessed the kinetics of IRF3 activation in LPS and poly(I:C)-stimulated bone marrow DCs. We analyzed the presence of its phosphorylated form in nuclear extracts (Fig. 2A) and performed immunofluorescence staining (data not shown). IRF3 activation was rapid and transient in response to LPS and poly(I:C). Because IRF1 was reported to mediate transcriptional activation of IL-27p28 in response to types I and II IFN, we then assessed the expression and activation of this transcription factor under our experimental conditions. We observed detectable IRF1 expression in resting DCs at the mRNA level (Fig. 2B). The expression of the IRF1 gene was further increased upon stimulation with LPS or poly(I:C). Immunoblot analysis and immunofluorescent microscopy experiments revealed that IRF1 is already present in the nucleus in unstimulated cells and further accumulates into the nucleus upon LPS or poly(I:C) stimulation (Fig. 2A, 2C). However, nuclear translocation of IRF1 induced by poly(I:C) was delayed compared with LPS. In the absence of autocrine type I IFN signaling, IRF1 translocation observed upon poly(I:C) stimulation was completely suppressed (data not shown). Surprisingly, we still observed a rapid but transient activation of IRF1 in LPS-stimulated IFNAR−/− DCs (Fig. 2E). Activation of the ISGF3 complex, revealed by nuclear localization of IRF9 and STAT2, was also observed in response to LPS and poly(I:C) (Fig. 2A).

Compared with IRF1, activation of ISGF3 was delayed in response to both stimuli, and it occurred earlier in response to LPS than to poly(I:C). Translocation of STAT2 assessed by immunofluorescence microscopy confirmed these results (Fig. 2D). We then analyzed the expression of IRF7, a gene known to be a direct target of ISGF3 (31). IRF7 mRNA levels gradually increased upon LPS or poly(I:C) stimulation (Fig. 2F). This induction was completely abrogated in absence of IRF9 (data not shown).

**FIGURE 1.** Type I IFN signaling is required for IL-27p28 expression induced by TLR ligation. A–D, DCs from C57BL/6 WT, IRF3−/− mice. E–G, DCs from SV129 WT, IFNAR−/− mice. DCs were incubated in medium alone or stimulated with optimal concentrations of LPS (100 ng/ml) or poly(I:C) (10 μg/ml). A and E, After 24 h of stimulation, supernatants were collected and analyzed for IL-27p28 level by ELISA. Results represent mean ± SEM of eight independent experiments for IRF3−/− and four independent experiments for IFNAR−/−. B, After 24 h of stimulation, supernatants were collected and analyzed for IFN-β level by ELISA. Results represent mean ± SEM of four independent experiments. p, 0.05; pp, 0.01; ppp, 0.001 compared with WT mice. C, D, F, and G, Total RNA was extracted and analyzed by real-time RT-PCR. Levels were normalized using β-actin mRNA as reference. One representative experiment of four is shown.
While IRF1 and IRF9 contribute to TLR-mediated expression throughout the time course (Fig. 3), assessed the possible contribution of IRF7 in Fig. 4 (I:C) was comparable in WT and IRF7 stimulation. IRF9 was critical for poly(I:C)-mediated (2–4 h poststimulation) rather than late time points (9–12 h post-stimulation). Both transcription factors seem to be important in the promotion of IL-27p28 mRNA accumulation at early thereafter (Fig. 3B). No differences were observed between the two groups, except for a modest decrease at late time points (9–12 h poststimulation) in poly(I:C)-treated IRF7−/− DCs (Fig. 4B, 4C). These results strongly suggest that ISGF3 directly promotes p28 gene activation. Interestingly, the residual expression of IL-27 observed in poly(I:C)-stimulated IRF3−/− DCs (Fig. 1) was abrogated in the absence of IRF3 and IRF7 (Supplemental Fig. 2). Hence, we cannot exclude that if IRF7 participates in poly(I:C)-mediated IL-27 production under these experimental conditions.

**IL-27p28 gene activation involves collaboration of IRF1 and IRF9 through direct recruitment to a proximal ISRE site**

Taken together, these experiments indicate that in response to LPS, early IRF1 activation occurs independently of type I IFN signaling. IRF3-dependent production IFNβ then leads to sustained activation of IRF1 and formation of the ISGF3 complex. In contrast, upon poly(I:C) stimulation, type I IFN production is mandatory for activation of both IRF1 and ISGF3. Finally, in response to both stimuli, ISGF3 complex allows upregulation of IRF7 expression. Kinetics of nuclear translocation of IRF1 in WT and IFNAR−/− DCs were assessed by immunofluorescent microscopy; the percentage of positive nuclei is shown. E, Kinetics of nuclear translocation of IRF1 and Stat2 were assessed by immunofluorescent microscopy in WT DCs. The percentage of positive nuclei is shown.
observed. We also confirmed that the low m.w. complex D contains IRF1 (lanes 5 and 10). Anti-Stat2 inhibited the migration of complex A (lanes 6 and 11), and no supershift was observed with nuclear extracts from IRF9−/− DCs (lane 14), strongly suggesting that this high m.w. complex contains ISGF3. The supershifts were found to be specific, because no supershift was observed with anti-SP1 Ab (lane 7). Although IRF7 was not identified as part of the complexes observed with nuclear extracts from stimulated DCs, we observed DNA–protein interaction when we incubated recombinant N-terminal IRF7 with the ISREp28wt probe (Fig. 5C). Competition experiments with an excess of different double-stranded oligonucleotides indicated that this binding was specific; it was inhibited in the presence of homologous unlabeled ISREp28wt or the ISRE element from the ISG15 promoter (ISG15WT), whereas it was unaffected by the mutated version of ISG15 (ISG15Mut) and ISREp28 (ISREp28MutAB) or by an irrelevant oligonucleotide.

These experiments indicate that upon LPS or poly(I:C) stimulation, high m.w. complexes containing IRF3 and/or ISGF3 and a low m.w. complex containing IRF1 physically interact with the p28 ISRE binding site. Although IRF7 also has the potential to bind to the p28 ISRE site, it did not contribute to the formation of the observed complex under our experimental conditions.

To determine whether IRF1 and ISGF3 are recruited to the endogenous IL-27p28 promoter region upon activation of DCs, we performed ChIP experiments using primers encompassing the ISRE site. As previously described (23), we found that IRF3 was recruited to the p28 promoter region (Fig. 5D). This event was already observed 90 min after stimulation. IRF1-specific recruitment was detectable in the absence of stimulation and was rapidly increased upon LPS stimulation. Upon poly(I:C) stimulation, IRF1 binding also increased, but at a later time point. Recruitment of IRF9 and STAT2 was detected 4 h after stimulation in response to LPS and poly(I:C). Therefore, these data indicate that TLR3- and TLR4-induced signaling leads to the recruitment of IRF3, IRF1, and ISGF3 to the endogenous p28 promoter region.

**FIGURE 3.** Differential role of IRF members in IL-27p28 gene activation in TLR3- and TLR4-stimulated DCs. DCs from WT, IRF1−/−, or IRF9−/− mice were incubated in medium alone or stimulated with LPS (100 ng/ml) or poly(I:C) (10 μg/ml). A. After 24 h of stimulation, supernatants were collected and analyzed for IL-27p28 level by ELISA. Results represent mean ± SEM of eight independent experiments. *p < 0.05; **p < 0.01 compared with WT mice. B and C. Total RNA was extracted and analyzed by real-time RT-PCR. One representative experiment of six is shown.

**FIGURE 4.** IRF7 is not involved in IL-27p28 gene expression. DCs from WT or IRF7−/− mice were incubated in medium alone or stimulated with LPS (100 ng/ml) or poly(I:C) (10 μg/ml). A. After 24 h of stimulation, supernatants were collected and analyzed for IL-27p28 level by ELISA. Results represent mean ± SEM of eight independent experiments. B and C. Total RNA was extracted and analyzed by real-time RT-PCR. One representative experiment of six is shown.
FIGURE 5. IRF1 and IRF9 are recruited to the p28 proximal promoter region. A, WT, IFNAR−/−, IRF1−/−, IRF9−/− or IRF7−/− DCs were incubated with medium alone, LPS, or poly(I:C) for 4 h. Nuclear extracts (10 μg) were subjected to EMSA using a radiolabeled ISREp28wt probe. Three inducible protein–DNA complexes (A, B, and D) were observed in WT DC conditions. B, Supershift experiment. Nuclear extracts were incubated with anti-IRF3, anti-IRF1, anti-Stat2, or anti-SP1 Abs. NS, nonspecific; SS, supershift. C, Recombinant N-terminal IRF7 (2 ng) was incubated with radiolabeled ISREp28wt probe in the absence or presence of 12.5-, 5-, and 50-fold excess of the indicated unlabeled competitor. D, ChIP experiments. DCs were incubated with medium alone or stimulated with LPS or poly(I:C). Chromatin samples were immunoprecipitated with anti-IRF3, anti-IRF1, anti-IRF9, or anti-Stat2 Abs. Purified DNA samples were subjected to real-time PCR amplification using primers specific for the IL-27p28 promoter region. Data were normalized using input as reference and were expressed as relative expression against the value obtained with control Abs. Results represent the mean ± SEM of triplicate experiments. E, Ectopic expression of IRF family members upregulates IL-27p28 promoter activity. HEK 293 cells were cotransfected with 400 ng of reporter plasmid, 100 ng of pRL-TK, and increasing amounts of the indicated IRF plasmid (10–800 ng). Results represent the mean ± SEM of three independent experiments performed in triplicate. F, HEK 293 cells were co-transfected with 400 ng of reporter plasmid, 100 ng of pRL-TK, and 400 ng of the indicated IRF plasmid. Results represent the mean ± SEM of four independent experiments performed in triplicate. Data are normalized against unstimulated WT promoter activity, which is set at 1. RLU, relative light unit.
We next examined the functional role of these IRF family members in the transcriptional regulation of the p28 gene. We cotransfected a (−1061/+13) IL-27p28 reporter plasmid with expression vectors encoding IRF1, a constitutively active form of IRF7 (IRF7[4D]) or a chimeric protein composed of IRF9 and the C-terminal region of Stat2 (IRF9-S2C) into HEK293 cells. As shown in Fig. 5E, each factor strongly transactivated the IL-27p28 promoter region. Similar results were obtained with the RAW macrophage cell line (data not shown). Under all of these conditions, the positive effect required an intact ISRE p28 site (Fig. 5E). Next, we looked for possible synergy among IRF1, IRF3, ISGF3, and IRF7. Combination of the IRF7(4D) plasmid with IRF1, IRF3(5D), or IRF9-S2C led to an increase in p28 promoter activity. However, the strongest activation was observed when IRF1 and IRF9-S2C were combined, indicating that these two transcription factors can cooperate for efficient IL-27p28 gene activation (Fig. 5F).

IRF1 and IRF9 contribute to production of IL27 by DCs upon in vivo exposure to microbial compounds

Our in vitro data highlight the critical role of a type I IFN loop in the control of IL-27 production in response to microbial compounds. We showed that this effect is mediated by the recruitment of IRF1 and IRF9 to a proximal ISRE site. Because possible redundancy with other pathways might occur in vivo, we wanted to assess their contribution to IL-27 production upon systemic exposure to microbial compounds. Upon LPS challenge, we observed that IRF1 was required for the initial production of IL-27 (2 h postinjection), whereas IRF9 was necessary to sustain IL-27 serum levels (4 h postinjection) (Fig. 6A). The effective production of IL-27 upon poly(I:C) administration was delayed compared with LPS (Fig. 6B). Serum levels were strongly reduced in IRF1- and IRF9-deficient mice under these experimental conditions. Because multiple cellular sources may account for IL-27 production in vivo, we sorted splenic CD11c+ DCs 2 h postchallenge, and we quantified p28 mRNA levels (Fig. 6C). In line with in vitro data and IL-27 production in the sera, induction of p28 mRNA in splenic DCs by LPS or poly(I:C) required IRF1 and IRF9. Finally, we also analyzed production of the two related cytokines, IL-12p70 and IL-23, in the sera of LPS-injected mice. IL-12p70 production was dependent on IRF1, but not IRF9, whereas IL-23 levels remained unaffected in the different strains (Supplemental Figs. 3, 4).

Discussion

Type I IFNs (α/β) are well known for their central role in antiviral responses. They are produced by most cells upon viral infection. They are also produced upon contact with viral or bacterial products by innate immune cells, such as DCs, upon activation of transcription factors from the IRF family in a cell-type and stimulus-dependent fashion (30, 32). In addition to classical IFN-stimulated genes involved in antiviral responses, type I IFN also regulates multiple aspects of innate and adaptive immune responses. The type I IFN autocrine–paracrine loop is also involved in the upregulation of surface costimulatory molecules (33, 34) and the amplification of cytokine production. Indeed, type I IFNs are required for optimal IL-12p35 expression (35). In agreement with other studies (24, 25), we showed herein that type I IFNs are also involved in the efficient production of the related cytokine IL-27. Type I IFNs have been linked to the pathogenesis of autoimmune disorders, such as systemic lupus erythematosus or type 1 diabetes. In sharp contrast, type I IFNs have proven to be beneficial in other clinical settings, such as multiple sclerosis (36). In the experimental autoimmune encephalomyelitis model, recent evidence indicates that the protective role of endogenous type I IFNs was mediated by the induction of IL-27 (37), which negatively regulated Th17-dependent immunity.

FIGURE 6. Critical role of IRF in IL-27 synthesis upon LPS and poly(I:C) challenge in vivo. A and B, WT, IRF1−/−, or IRF9−/− mice (15–35 mice per group) were injected i.p. with PBS, LPS (10 mg/kg), or poly(I:C) (2.5 mg/kg). After the indicated time, blood was collected, and sera were assayed for IL-27p28 levels by ELISA. Results are expressed as mean ± SEM. C, CD11c+ spleen cells were isolated 2 h after injection (8–20 mice per group). Total RNA was extracted and analyzed by real-time RT-PCR. Results are expressed as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. ns, nonsignificant compared with WT mice.

FIGURE 7. A two-step activation model of the IL-27 p28 gene. See Discussion for more details.
inflammation (8–10). These results highlight the in vivo relevance of a type I IFN-IL-27 axis. In the present work, we defined at the molecular level how TLR and type I IFN signaling cooperate, through activation of multiple members of the IRF family for efficient IL-27p28 gene activation.

We observed different patterns of IL-27 gene activation in response to LPS or poly(I:C). Activation of the p28 gene by LPS is rapid and transient. In comparison, activation by poly(I:C) is delayed, less intense, but sustained. The initial burst of transcription induced by LPS is strongly dependent on IRF1 and IRF3. In response to poly(I:C), significant, but delayed, expression of IL-27 is still observed in the absence of these transcription factors but not in the absence of IRF9. These results suggest a two-step process. In the absence of IRF3, IRF7 directly or indirectly participates in poly(I:C)-mediated p28 induction. There are several differences in the LPS and poly(I:C) signaling pathways that could account for these observations. In agreement with previous reports, we found that the rapid translocation of IRF1 in response to LPS requires MyD88, a pathway not activated in response to poly(I:C) [(38) and our own unpublished results]. Conversely, sustained p28 activation in response to poly(I:C) could implicate non-TLR pathways, because dsRNA also triggers MDA5/IFN-β promoter stimulator 1 activation (39, 40). Furthermore, other members of the IRF family could participate in the transcriptional regulation of the p28 gene. For example, IRF8 plays an important role in the expression of IL-12p35 and p40 genes (41, 42). This factor also contributes to the amplification of type I IFN production in DCs (43). Preliminary experiments indicated that the p28 promoter is not responsive to IRF8 overexpression. However, we cannot exclude a direct or indirect contribution of endogenous IRF8 to the control of the gene in DCs.

Based on our experimental data, we propose the following model for activation of the IL-27p28 gene in response to TLR4 or TLR3 (Fig. 7). Triggering of the TRIF-dependent pathway leads to IRF3 activation, which is recruited to the p28 promoter region. This pathway also leads to the rapid production of type I IFN, such as IFN-β. If the MyD88 pathway is also activated, as is the case upon TLR4 ligation, IRF1 rapidly translocates to the nucleus and cooperates with IRF3 for the initial induction of the IL-27p28 gene. Once IFN-β is secreted, autocrine signaling leads to upregulation of IRF1 expression, sustained activation of this transcription factor, and formation of the ISGF3 complex, which is also recruited to the p28 ISRE site and is critical for amplification of IL-27p28 gene expression. ISGF3 activation leads to the expression of IRF7, which, in some settings, could also participate in late activation of the p28 gene directly or through amplification of type I IFN synthesis.

It was demonstrated that autocrine type I IFNs contribute to activation of the IL-12p35 gene (35). Indeed, IRF1 and IRF3 were shown to participate in the transcriptional regulation of IL-12p35 gene expression in response to IFN-γ or TLR stimulation (28, 41, 44). We confirm the implication of IRF1 in LPS-mediated induction of the IL-12p35 gene in vitro and in vivo (Supplemental Fig. 5). In sharp contrast, IRF9 was found to be dispensable for the expression of this gene and was not recruited to the promoter region upon TLR stimulation. These results indicate that the positive effect of autocrine type I IFN signaling on the IL-12p35 gene is mediated by the upregulation of IRF1 and not by formation of the ISGF3 complex, revealing that activation of the closely related IL-27p28 and IL-12p35 subunits requires different combinations of IRF family members.

Finally, LPS-mediated expression of IL-23p19 and the production of IL-23 were found to be independent of IRF1 and IRF9 (Supplemental Fig. 4). Taken together, these data indicate that the balance among IL-12, IL-23, and IL-27 production is strongly dependent on the set of IRF family members that are activated upon contact with specific pathogen-associated molecular patterns. We suggest that these findings could help to design novel therapies based on endogenous IL-27.

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Disclosures
The authors have no financial conflicts of interest.

References


**Legends to Supplementary Figures.**

**FIGURE S1. TRIF-dependent signaling is required for IL-27p28 expression induced by TLR ligation.** DCs from WT or LPS2 (TRIF\(^{-/-}\)) mice were incubated in medium alone or stimulated with LPS (100 ng/ml) or polyI:C (10µg/ml). A, After 24h of stimulation, supernatants were collected and analyzed for IL-27p28 level by ELISA. Results represent mean ± SEM of six independent experiments. ***, p <0.001 as compared with WT mice. B and C, Total RNA was extracted and analyzed by real-time RT-PCR. One representative experiment of four is shown.

**FIGURE S2. In absence of IRF3, IRF7 contributes to production of IL-27p28 in response to polyI:C.** DCs from WT, IRF3/7\(^{-/-}\) mice were incubated in medium alone or stimulated with optimal concentrations of LPS (100 ng/ml) or polyI:C (10µg/ml). A, After 24h of stimulation, supernatants were collected and analyzed for IL-27p28 level by ELISA. Results represent mean ± SEM of four independent experiments. B, After 24h of stimulation, supernatants were collected and analyzed for IFNβ level by ELISA. Results represent mean ± SEM of four independent experiments. ***, p <0.01 as compared with WT mice. C and D, Total RNA was extracted and analyzed by real-time RT-PCR. Levels were normalized using β-actin mRNA as reference. One representative experiment of four is shown.

**FIGURE S3. Differential role of IRF in IL-12 synthesis upon LPS stimulation.** A, DCs from WT, IRF1\(^{-/-}\) or IRF9\(^{-/-}\) mice were incubated in medium alone or stimulated with LPS (100 ng/ml) or polyI:C (10µg/ml). Total RNA was extracted and analyzed by real-time RT-PCR. One representative experiment of six is shown. B, WT, IRF1\(^{-/-}\) or IRF9\(^{-/-}\) mice (8 to16 mice per group) were injected i.p. with PBS, LPS (10 mg/kg) or polyI:C (2,5 mg/kg). After the indicated time, blood was collected and sera were assayed for IL-12p70 levels by ELISA.
Results are expressed as mean ± SEM. C, CD11c+ spleen cells were isolated 2h after injection (8 to 20 mice per group). Total RNA was extracted and analyzed by real-time RT-PCR. Results are expressed as mean ± SEM. *, p <0.05; and **, p < 0.01; ns, non significant as compared with WT mice. D, ChIP experiments. DCs were either incubated with medium alone or stimulated with LPS or polyI:C. After stimulation, cells were treated with 1% formaldehyde to cross-link proteins bound to DNA. After sonication, chromatin samples were immunoprecipitated with anti-IRF1 Abs or Stat2 Abs. Purified DNA samples were subjected to PCR amplification using primers specific for IL-12p35 promoter region. For each sample, 10% of the cross-link-released chromatin was saved and used as input control.

FIGURE S4. IL-23 synthesis upon LPS stimulation is not dependent on IRF members. A, DCs from WT, IRF1−/− or IRF9−/− mice were incubated in medium alone or stimulated with LPS (100 ng/ml) or polyI:C (10µg/ml). Total RNA was extracted and analyzed by real-time RT-PCR. One representative experiment of six is shown. B, WT, IRF1−/− or IRF9−/− mice (8 to 19 mice per group) were injected i.p. with PBS, LPS (10 mg/kg) or polyI:C (2,5 mg/kg). After the indicated time, blood was collected and sera were assayed for IL-23 levels by ELISA. Results are expressed as mean ± SEM.
Supplementary Figure 1

A. mIL-27p28

B. mIL-27p28 mRNA

C. mIL-27p28 mRNA
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4

A) mIL-23p19 mRNA

B) mIL-23

WT
IRF1 -/-
IRF9 -/-

LPS treatment

LPS injection
<table>
<thead>
<tr>
<th>Oligonucleotides, 5'-3'</th>
<th>Real-time RT-PCR</th>
<th>ChIP</th>
<th>ChIP - real time PCR</th>
</tr>
</thead>
</table>
| beta-actin              | Forward TCCTGAGCGCAAGTACTCTGT  
Reverse CTGATCCACATCTGCTGGAAG  
Probe 6FAM-ATCGGTGGCTCCATCCTGGC-BHQ1 |  |  |
| IL-27p28                | Forward ATCTCGATTGCCAGGAGTGA  
Reverse GTGTTAGCGAGGAAGCAGAGT  
Probe 6FAM-TTCCCAATGTCCCTGACTTTCCA-BHQ1 |  |  |
| IL-12p35                | Forward CTTAGCCAGTCCCCGAACCT  
Reverse TTGGTCCCAGTGTAGTCTT  
Probe 6FAM-TTCCCAATGTCCCTGACTTTCCA-BHQ1 |  |  |
| IL-23p19                | Forward CCCGTATCCAGTGTGAGATG  
Reverse CCGTTGAAGATGTCCAGTCA  
Probe 6FAM-CCACAAGGACTCAAGGACAAGGAC-BHQ1 |  |  |
| IRF1                    | Forward TCGTCAGCAGCAGTCTCTCT  
Reverse TTTGGCTATCTCTCCCTTCT  
Probe 6FAM-TCCGAAGCCGCAACAGACGA-BHQ1 |  |  |
| IRF7                    | Forward CCAGTTGATCCGCAATAGGT  
Reverse TGTGTTGGCGAGCACTT  
Probe 6FAM-CGAACCTTAGCGAGCTTGGA-BHQ1 |  |  |
| ChIP IL-12p35 promoter  | Forward TTGGCTTTGCTCTGAGTGTG  
Reverse GCTGACCTTTGGGAGACAT  |  |  |
| ChIP - real time PCR IL-27p28 promoter | Forward CCCTCTGGGAAGGAAATTA  
Reverse CCTCTGTGTCAGCAGCATCT  
Probe 6FAM-TGCGACCCAGTTTCTACTTTCTGTC-BHQ1 |  |  |