Regulation of IL-27 p28 gene expression in macrophages through MyD88- and interferon-γ–mediated pathways

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Interleukin (IL)-27 is the newest member of the IL-12 family of heterodimeric cytokines composed of the Epstein-Barr virus–induced gene 3 and p28 chains. IL-27 not only plays an important role in the regulation of differentiation of naive T helper cells but also possesses antiinflammatory properties. IL-27 is an early product of activated monocytes/macrophages and dendritic cells. However, the mechanisms whereby inflammatory signals stimulate IL-27 production have not been explored. In this study, we investigated the transcriptional regulation of the mouse IL-27 p28 gene in macrophages in response to lipopolysaccharide (LPS) and interferon (IFN)-γ. We found that LPS-stimulated p28 production was completely dependent on the Toll-like receptor 4/myeloid differentiation factor 88 (MyD88)–mediated pathway but only partially dependent on nuclear factor κB c-Rel. IFN-γ–induced p28 production/secretion was also partially dependent on MyD88 but independent of c-Rel. We then cloned the mouse p28 gene promoter and mapped its multiple transcription initiation sites. Furthermore, we identified critical promoter elements that mediate the inductive effects of LPS and IFN-γ, separately and synergistically, on p28 gene transcription in a c-Rel– and interferon regulatory factor 1–dependent manner, respectively.

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Abbreviations used: BMDM, bone marrow–derived macrophages; ChIP, chromatin immunoprecipitation; EBI3, Epstein-Barr virus–induced gene 3; EMSA, electrophoretic mobility shift assay; IRF, interferon regulatory factor; mRNA, messenger RNA; MyD88, myeloid differentiation factor 88; qPCR, real-time quantitative PCR; RLM-RACE, 5′ RNA ligase-mediated rapid amplification of cDNA end; TIS, transcription initiation site; TLR, Toll-like receptor.

IL-27, a novel IL-12 family cytokine, is a heterodimeric molecule composed of Epstein-Barr virus–induced gene 3 (EBI3), an IL-12 p40-related protein, and p28, an IL-12 p35-related polypeptide (1). IL-27 is produced early by activated antigen-presenting cells in response to microbial infection. It is able to induce clonal proliferation of naive but not memory $CD4^+$ T cells and synergizes with IL-12 in IFN-γ production by naive $CD4^+$ T cells (1). IL-27 plays an important role in autoimmune disease and host defense against infection. IL-27 receptor/WSX-1 knockout mice show increased susceptibility to intracellular pathogens such as *Leishmania major* (2) and *Listeria momocytogenes* (3) because of impaired IFN-γ production from $CD4^+$ T cells. IL-27 p28 (abbreviated as p28 hence forth) is highly expressed in inflammatory bowel diseases (4, 5) and experimental autoimmune encephalomyelitis (6). Neutralizing the p28 subunit suppressed the ongoing adjuvant-induced arthritis (7). Recent studies, however, have shown that IL-27/WSX-1 signaling also negatively regulates the inflammatory processes. Exacerbation of experimental allergic asthma was observed in WSX-1–deficient mice through affecting the Th cell differentiation into the Th1 or Th2 linage (8). IL-27 inhibits CD28-mediated IL-2 production through suppressor of cytokine signaling 3 (9, 10). Studies in *Toxoplasma gondii* (11), *Trypanosoma cruzi* infection (12), and concanavalin A–induced hepatitis (13) have demonstrated that WSX-1 plays a role in limiting the intensity and duration of T cell activation. IL-27 receptor–deficient mice chronically infected with *Toxoplasma gondii* developed severe neuroinflammation that was $CD4^+$ T cell dependent and was associated with a prominent IL-17 response. In vitro, treatment of naive primary T cells with IL-27 suppressed the development Th-17 cells induced by IL-6 and transforming growth factor-β (14). IL-27Rα–deficient mice were also hypersusceptible to experimental autoimmune encephalomyelitis and generated more IL-17–producing Th cells (15).

It has been somewhat of an enigma why and how IL-27 exerts both inflammatory and anti-inflammatory effects on T cells. A recent study provides an answer, which lies in the ability of IL-27 to stimulate both STAT1 and STAT3 in naive Th cells while stimulating

only STAT3 in activated Th cells (16). This differential ability of IL-27 explains why IL-27 can activate naive Th cells whereas it turns inhibitory on activated Th cells. It would be interesting to further identify the molecular mechanism underlying the differential activation of STA1 and STAT3 by IL-27 in naive versus activated Th cells.

In addition, recent studies indicated that IL-27 has potent antitumor effects. The antitumor activity of IL-27 against colon carcinoma and neuroblastoma is mainly dependent on $CD8^+$ T cell, IFN-γ, and T-bet (17–20). And the anti-B16 melanoma effect of IL-27 seems to act through suppressing angiogenesis (21).

Microbial infection of mammals typically triggers host responses through numerous pathogen recognition receptors that sense pathogen-associated molecular patterns conserved in a large number of microorganisms. One family of such pathogen recognition receptors is the Toll-like receptors (TLRs). TLR4, in particular, is a very important receptor that can detect LPS derived from the cell wall of gram-negative bacteria. Upon recognition of LPS by TLR4 expressed on macrophages in conjunction with CD14, several intracellular signaling molecules and adaptors such as myeloid differentiation factor 88 (MyD88) are recruited to the ligand–receptor complex, triggering a downstream signaling cascade of events leading to the activation of a multitude of cytoplasmic kinases such as mitogen-activated protein kinase and nuclear transcription factors such as NF-κB and resulting in the production of several proinflammatory cytokines such as IL-1, TNF-α, IL-6, and IL-12 that drive the host response to the invading pathogens (22).

IFNs are widely expressed cytokines that are the frontier of host defense against infections and have important roles in immunosurveillance for malignant cells (23). IFN-γ is a type II interferon produced mainly by NK, NKT, $CD4^+$ T cell, and $CD8^+$ cytotoxic T cell. IFN-γ is essential for mounting a cell-based immune response and promotes protection against the intracellular pathogens such as mycobacteria, but also plays a key role in the chronification of inflammatory responses such as atopic dermatitis, rheumatoid arthritis, and systemic lupus erythematosus (23). IFN-γ is also essential in immunosurveillance against malignant transformation (24). IFN-γ acts on a remarkable range of distinct cell populations including immune cells and nonimmune cells. Of these, macrophages are among the most important. IFN-γ activates direct microbicidal functions of macrophages and promotes antigen processing and presentation capacities of macrophages (25). The impact of IFN-γ on macrophage phenotype and function is achieved by a profound alteration of the macrophage transcriptional program in response to IFN-γ. It has been estimated that exposure to IFN-γ results in changes in expression of ∼25% of the mouse genome (26). Interferon regulatory factor (IRF)-1 is the major member of the IRF family of transcription factors activated by IFN-γ and is essential for many IFN-γ responses.

The IRF family consists of nine mammalian transcription factors (IRF-1 to 9) that commonly possess a unique helix-turn-helix DNA-binding motif (27). The first discovered member of this family, IRF-1, has a remarkable functional diversity in the regulation of cellular responses in host defense. IRF-1 targets different sets of genes in various cell types in response to diverse cellular stimuli and evokes appropriate innate and adaptive immune responses (27). It has been firmly established as a critical effector molecule in IFN-γ–mediated signaling and in the development and function of NK, NKT, and cytotoxic T lymphocytes (28–33). IRF-1 also has direct anti-proliferative effects, thus acting as a tumor suppressor and tumor susceptibility gene (34).

Given the importance of IL-27 in regulating host response to both foreign and endogenous threats and the total lack of knowledge about how this cytokine is regulated, we undertook the present study to investigate the molecular mechanisms that regulate p28 subunit gene expression in macrophages activated by bacterial LPS and IFN-γ with a focus on their intracellular and nuclear effectors. This study revealed that MyD88, NF-κB c-Rel, and IRF-1 play varying roles in mediating the inductive effects of LPS and IFN-γ on p28 gene expression at the transcriptional level.

RESULTS

IL-27 p28 expression is regulated by IFN-γ and LPS

We measured p28 protein and messenger RNA (mRNA) expression in thioglycolate-elicited mouse peritoneal macrophages treated with IFN-γ, LPS, or IFN-γ priming plus LPS in a kinetic manner. The data showed that whereas IFN-γ alone stimulated p28 protein secretion kinetically (Fig. 1 A) LPS induced ∼10-fold greater levels of p28 in a kinetics faster than that of IFN-γ stimulation (Fig. 1 B). Treatment with IFN-γ and LPS further increased the p28 level about fivefold compared with LPS alone (Fig. 1 B). The levels of mRNA expression induced by IFN-γ or LPS by and large paralleled those of protein production as measured by regular RT-PCR (Fig. 1 C) or by real-time quantitative PCR (qPCR; (Fig. 1 D), suggesting that p28 expression is primarily regulated at the mRNA level.

LPS-induced p28 expression is mediated through MyD88–NF-κB signal transduction pathway

To explore the LPS signal transduction pathway in the regulation of IL-27 p28 expression, we first investigated the kinetic expression pattern of p28 mRNA induced by LPS. The data showed LPS-induced p28 mRNA expression occurred as early as 1 h and reached the peak around 8 h, followed by a decline after 16 h (Fig. 2 A). It is well established that the TLR4 ligand LPS can activate downstream genes through both MyD88-dependent and -independent pathways. Thus we examined the role of MyD88 in LPS-induced p28 gene expression. We treated the mouse peritoneal macrophages elicited from WT and $MyD88^{-/-}$ mice with IFN-γ, LPS, or IFN-γ plus LPS for 24 h and measured p28 protein and mRNA expression by ELISA and qPCR, respectively. LPS induced p28 protein (Fig. 2 B) and mRNA (Fig. 2 C) expression was almost totally abolished in $MyD88^{-/-}$ cells, whereas

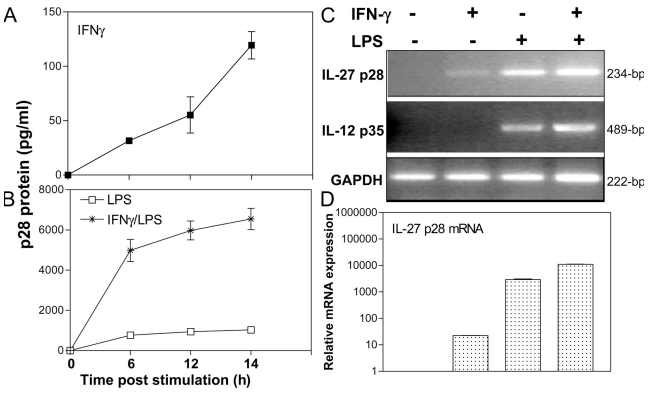

Figure 1. Expression of p28 protein and mRNA in macrophages. The production of IL-27 p28 was measured by ELISA from cell-free supernatants of mouse peritoneal macrophage cultures (0.5×10^6 cells in 1 ml) stimulated with IFN-γ (A) and LPS (B) or primed with IFN-γ for 16 h followed by LPS stimulation (B) for the indicated times in hours. Results shown in A and B represent the mean plus SE of three independent experiments. Total RNA was isolated from these macrophages and subjected to both semi-quantitative (C) and quantitative real-time (D) PCR analyses for p28 mRNA expression. Data was normalized relative to GAPDH mRNA expression levels in each respective sample and further normalized to the sample from untreated cells, which was set as 1 (D).

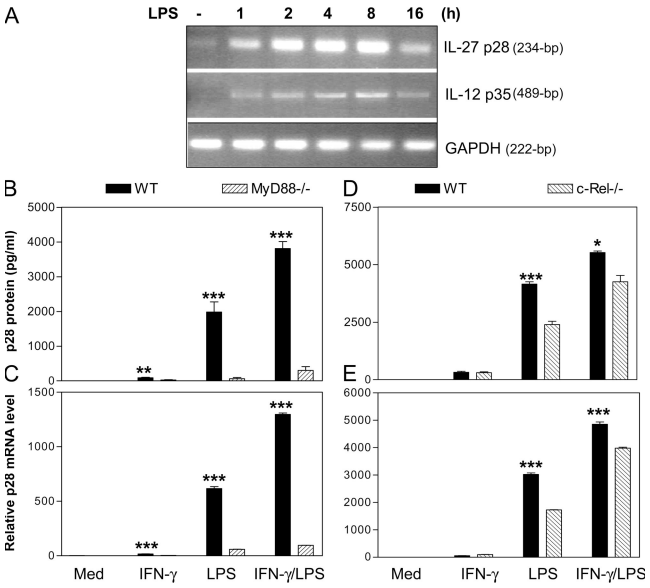

Figure 2. Role of MyD88 and NF-κB in p28 expression. (A) Kinetic expression of p28 mRNA in LPS-treated macrophages. Total RNA was isolated from mouse peritoneal macrophages treated with LPS at different time points and subjected to semi-quantitative PCR. GAPDH mRNA expression from the same samples was measured as a loading control. The production of p28 was measured by ELISA from cell-free supernatants of MyD88$^{-/-}$ (B) and c-Rel$^{-/-}$ (D) with their control WT macrophages (0.5×10^6 cells in 1 ml) stimulated with IFN-γ and LPS or IFN-γ plus LPS stimulation for 24 h. Results shown in B and D represent the mean plus SE of four mice per group. RNA was extracted from peritoneal macrophages of WT, MyD88$^{-/-}$ (C), and c-Rel$^{-/-}$ (E) mice treated with IFN-γ, LPS, or IFN-γ plus LPS for 6 h and subjected to qPCR analysis for p28 mRNA expression. Data represented pooled RNA from four mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

IFN-γ-induced p28 expression was partially decreased. NF-κB plays a vital role in LPS-induced production of many proinflammatory cytokines. In particular, c-Rel has been shown to be essential for IL-12 p35 gene transcription (35). To determine the role of c-Rel in the transcriptional induction of the p28 gene, which is homologous to p35, we treated the c-Rel$^{-/-}$ macrophages and its WT control with IFN-γ and LPS or IFN-γ plus LPS. The data showed that LPS-induced p28 protein (Fig. 2 D) and mRNA (Fig. 2 E) decreased by ~50% compared with WT control, whereas IFN-γ–induced p28 expression was comparable between WT and c-Rel$^{-/-}$ cells (Fig. 2, D and E). The combined effect of IFN-γ and LPS on p28 expression reflected the partial dependence of LPS and independence of IFN-γ on c-Rel, respectively (Fig. 2, D and E). These results indicate that LPS-induced p28 gene expression is largely dependent on the MyD88-mediated pathway, whereas IFN-γ–induced p28 expression is only partially dependent. Furthermore, c-Rel appears to be partially required for LPS-induced p28 expression whereas IFN-γ–induced p28 expression does not require c-Rel.

Expression of p28 mRNA and protein is impaired in IRF-1–deficient macrophages

Our previous study demonstrated that IFN-γ–regulated IL-12 p35 expression production requires IRF-1 (36). To determine the role of IRF-1 in IFN-γ–induced p28 gene expression, mouse peritoneal macrophages elicited from WT or IRF-1$^{-/-}$ mice were stimulated with IFN-γ, LPS, or IFN-γ plus LPS; cell-free culture supernatants were collected and subjected to ELISA for p28 protein secretion; and RNA was isolated and subjected to qPCR analysis. The data shows that both p28 protein secretion (Fig. 3 A) and mRNA expression (Fig. 3 B) by IRF-1$^{-/-}$ macrophages stimulated by IFN-γ were completely abrogated. Interestingly, LPS-stimulated p28 production (Fig. 3 A) and mRNA expression (Fig. 3 B) was also reduced by ~85%. IFN-γ plus LPS–induced p28 expression was also decreased in IRF-1$^{-/-}$ macrophages (Fig. 3, A and B).

To gain further understanding of the mechanism of IFN-γ–mediated induction, we analyzed the kinetic expression pattern of p28 mRNA in IFN-γ–treated macrophages from WT and IRF-1$^{-/-}$ mice. In WT macrophages, IFN-γ–induced p28 mRNA expression appeared at 3 h after IFN-γ treatment and reached the peak at 6 h, and then declined, whereas it failed to induce p28 expression in IRF-1$^{-/-}$ macrophages at most time points except for the 12-h point (Fig. 3 C). To confirm the inductive role of IRF-1 on p28 gene expression in primary macrophages, we performed a gene delivery experiment using adenovirus-expressing IRF-1 that we previously established (37). As shown in Fig. 3 D, the IRF-1 adenovirus-transduced mouse peritoneal macrophages displayed increased p28 mRNA expression (Fig. 3 D, top) in a dose-dependent manner in correlation with increased IRF-1

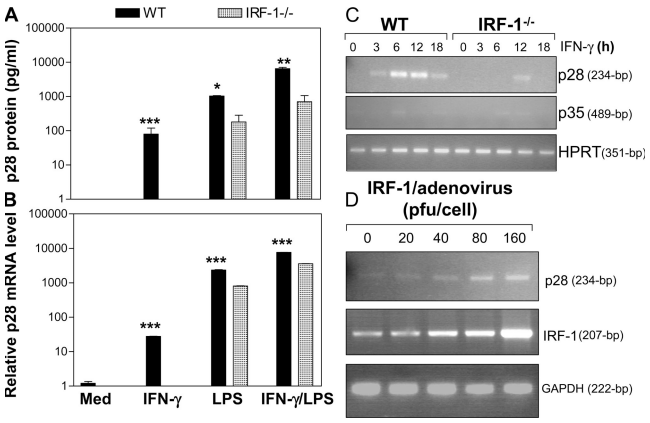

Figure 3. Role of IRF-1 in p28 gene expression. (A) Supernatant from WT and IRF-1$^{-/-}$ peritoneal macrophage culture treated with IFN-γ and LPS or IFN-γ plus LPS for 24 h were analyzed for p28 production by ELISA. Data represents mean plus SD from four mice. (B) Total RNA was extracted from WT and IRF-1$^{-/-}$ peritoneal macrophages treated with IFN-γ, LPS, and IFN-γ plus LPS for 6 h and analyzed by qPCR for p28 mRNA expression. Data was normalized relative to GAPDH mRNA expression levels of untreated WT cells in each respective sample. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Kinetic expression of p28 mRNA in IFN-γ–treated WT and IRF-1$^{-/-}$ macrophages. RNA was extracted from peritoneal macrophages of WT and IRF-1$^{-/-}$ mice treated with IFN-γ at different times as indicated and analyzed by RT-PCR for p28 mRNA expression. HPRT mRNA expression from the same samples was measured as a loading control. (D) WT mouse peritoneal macrophages were transduced with IRF-l/adenovirus at different doses of the virus from 20 to 160 PFU per cell for 48 h. Total RNA was extracted from the transduced macrophages and subjected to RT-PCR for p28 mRNA expression (top). The expression of IRF-1 mRNA was also measured from the same cDNA to verify the transduction efficiency (middle). GAPDH mRNA expression from the same samples was measured as a loading control (bottom).

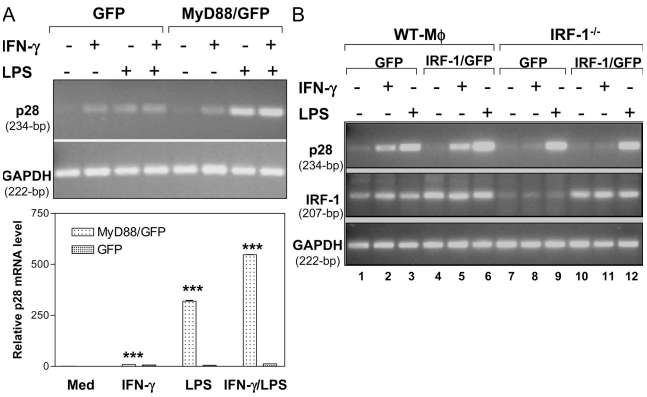

Figure 4. Reconstitution of MyD88 and IRF-1 in MyD88$^{-/-}$ and IRF-1$^{-/-}$ BMDM. (A) *MyD88*$^{-/-}$ BMDM were infected with retroviruses encoding MyD88 or GFP and treated with IFN-γ, LPS, and IFN-γ plus LPS for 5 h. Total RNA was extracted from the transduced BMDM and analyzed for p28 and GAPDH mRNA expression by semi-quantitative PCR (top) and real-time PCR (bottom). Data are one representative of two experiments with similar results (***, $P < 0.001$). (B) WT and *IRF-1*$^{-/-}$ BMDM were infected with lentiviruses encoding IRF-1/GFP or GFP alone and treated with IFN-γ and LPS for 5 h. Total RNA was extracted from the transduced BMDM and analyzed for p28 (top), IRF-1 (middle), and GAPDH (bottom) mRNA expression by semi-quantitative PCR.

mRNA expression (Fig. 3 D, middle). The control LacZ adenovirus-transduced cells did not express p28 (unpublished data). Collectively, these results strongly demonstrate that IRF-1 is an essential transcription factor for p28 gene expression induced by IFN-γ, and to a lesser extent by LPS, in primary macrophages.

Reconstitution of MyD88 and IRF-1 expression in gene-deficient macrophages

To confirm the role of MyD88 and IRF-1 in p28 gene expression we reconstituted their expression in MyD88- and IRF-1–deficient bone marrow–derived macrophages (BMDM) by retrovirus and lentivirus transduction, respectively. MyD88-deficient BMDM were transduced with retroviral vectors expressing GFP or MyD88/GFP (38). As show in Fig. 4 A, MyD88$^{-/-}$ BMDM had greatly diminished response to LPS, which, after reconstitution, was strongly enhanced compared with GFP retrovirus–transduced cells, confirming the role of MyD88 in LPS-stimulated p28 expression. In contrast, reconstitution of IRF-1 expression in IRF-1–deficient BMDM (Fig. 4 B) did not result in the restoration of IFN-γ response (Fig. 4 B, lanes 10–12), suggesting that additional, developmentally generated factors may be required for rescuing p28 expression.

Defining the p28 promoter by mapping the transcription initiate site

To determine whether IFN-γ–induced p28 expression is at the transcriptional level, we measured nascent p28 primary transcripts by RT-PCR using a pair of oligonucleotide primers corresponding to intron1 and exon2 of the mouse p28 gene, respectively. As shown in Fig. 5 A, IFN-γ induced the p28 primary transcripts kinetically starting around 2 h (Fig. 5 A, top). The p28 mRNA expression induced by IFN-γ basically followed the primary transcript expression pattern (Fig. 5 A, middle). These data suggests that IFN-γ–induced p28 gene expression is primarily exerted at the level of transcription.

To further study transcriptional regulation of the p28 gene we decided to define its promoter region (see Materials and methods for details) and to map its transcription initiation sites (TISs). Total RNA generated from nontreated and IFN-γ–treated mouse macrophages were used to map the TIS of the p28 gene by the method of 5′ RNA ligase-mediated rapid amplification of cDNA end (RLM-RACE). Out of the 20 resultant clones analyzed, five sequences were identified that differed in the sequence at the 5′ end. Thus, these different 5′ sequences defined five TISs in the *p28* gene. Fig. 5 C shows the sequence of the p28 promoter with these five TISs and some putative transcription factor-biding sites indicated. To determine the relative importance of these five TISs, we performed RT-PCR using a common antisense primer and distinct sense primers corresponding to the unique 5′ ends of the five TISs. As shown in Fig. 5 D, IFN-γ treatment

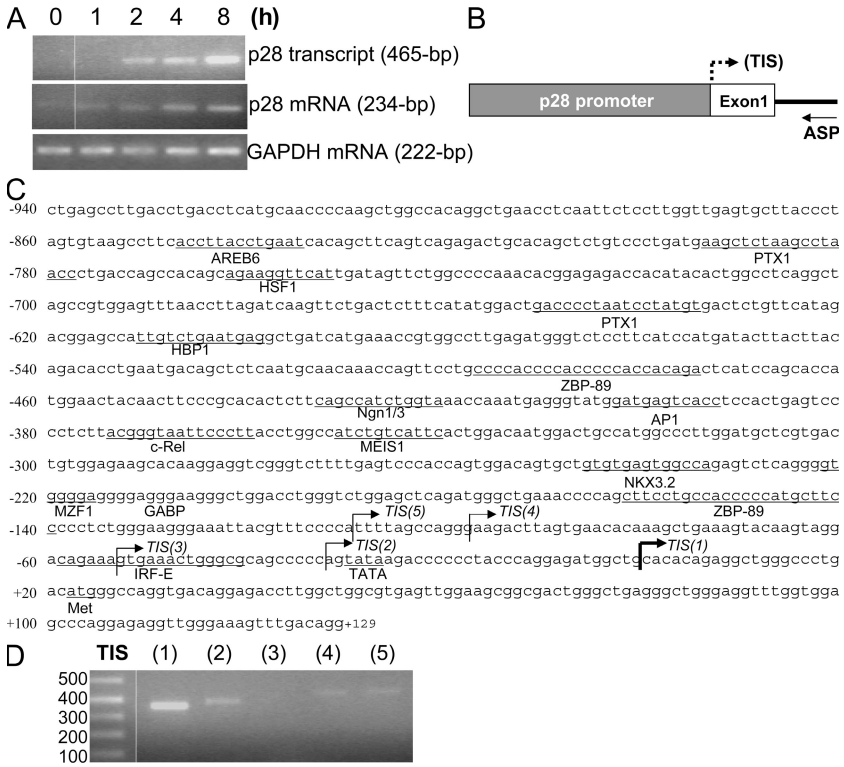

Figure 5. Mapping the IL-27 p28 promoter and the TISs. (A) Nascent p28 transcript and steady-state p28 mRNA expression. Total RNA was extracted from WT peritoneal macrophages treated with IFN-γ for different hours as indicated and reverse transcribed into cDNA using random primers. For p28 transcripts (top), a pair of primers flanking intron1 and exon2 were used for amplification by PCR. For steady-state p28 (middle) and GAPDH (bottom) mRNA amplification, the primers were derived from exons only. Data represents one of three experiments with similar results. (B) Schematic of the p28 promoter with the hypothetical TIS marked by a dashed arrow. The antisense primer (ASP) used in the RLM-RACE procedure corresponds to a segment in intron1. (C) Sequence of mouse *p28* promoter that we cloned containing some putative transcription factor binding sites predicted by MatInspector and the five mapped TISs (arrows). The promoter coordinates (from −940 down to +129) are relative to the most dominant TIS(1) (thick arrow) located at +1. (D) Relative expression levels of transcripts initiated from the five different p28 TISs. Total RNA was extracted from WT peritoneal macrophages–treated IFN-γ (8 h) and 1 μg RNA was reverse transcribed into cDNA, which was then used to identify the p28 TIS by RLM-RACE. A common antisense primer and five distinct sense primers were used to amplify the differentially initiated p28 transcripts (mRNA) by PCR.

induced predominantly a transcript that originated from TIS(1). Thus, TIS(1) is designated +1 of the p28 promoter and the sequence upstream of +1 is regarded as the p28 promoter region. In addition, we found that LPS treatment induced a differential TIS usage pattern identical to that of IFN-γ treatment (unpublished data).

Mechanism of LPS-stimulated p28 gene transcription

We were interested in elucidating the way that LPS stimulates p28 gene expression. To that end we examined the LPS response of the initial p28 gene promoter (−940/+129) that we cloned. To our surprise, this promoter construct was not responsive to LPS in the transient transfection system in RAW264.7 cells (unpublished data). We then extended the promoter region up to −3,076, and found that this longer promoter became responsive to LPS while also highly responsive to IFN-γ (Fig. 6 A). Sequential 5′ deletion of this construct down to −739 resulted in strong loss of LPS response, and to a lesser degree, of IFN-γ response, and of the synergistic response to both LPS and IFN-γ (Fig. 6 A). This result indicated that there are at least two LPS response elements, one residing between −3,076 and −1,222 and one between −1,222 and −835.

Because the transcriptionally inductive effects of LPS are primarily mediated by NF-κB, we scanned the promoter region and identified four putative NF-κB binding sites localized at –3,051/–3,042 (NF-κB #1), –1,230/–1,221 (NF-κB #2), –1,189/–1,178 (NF-κB #3), and –1,146/–1,137 (NF-κB #4). We then mutated each one of them and found that only mutations in the #1 element (mutant 1) lost a significant portion (∼40%) of its LPS response (Fig. 6 B). Furthermore, when we cotransfected the −3,076/+129 p28 promoter-reporter construct with an IκB-α mutant expression vector, which blocks the phosphorylation of the endogenous IκB-α and the release of NF-κB from the NF-κB–IκB complex resulting in suppression of the NF-κB nuclear translocation (39), both LPS- and IFN-γ–induced p28 promoter activity was abrogated (Fig. 6 C). This result

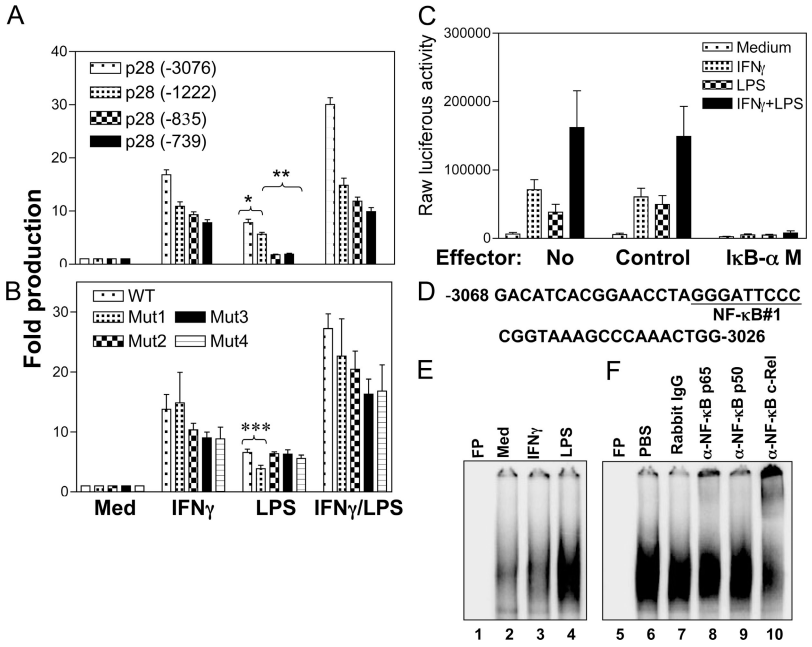

Figure 6. LPS response of p28 promoter constructs. (A) The −3,076/+129 and various 5′ truncation constructs of the mouse p28 promoter-luciferase reporter were transiently transfected into RAW264.7 cells by electroporation and the transfected cells were treated with 10 ng/ml IFN-γ, 1 μg/ml LPS, or IFN-γ plus LPS for 7 h. Luciferase activity from each construct was measured from cell lysates and normalized to the activity obtained with the untreated condition as relative activities (fold induction). Results shown are mean plus SE of six independent experiments. (B) RAW264.7 cells were transiently transfected with WT and the four putative NF-κB–RE mutant *p28* promoter constructs. Transfected cells were treated with IFN-γ, LPS, or IFN-γ plus LPS for 7 h. Luciferase activity was measured from the cell lyses and normalized to the activity of medium alone as relative activities (fold induction). Data represent the mean ± SE from six independent experiments. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$, comparing between indicated groups. (C) RAW264.7 cells were transiently cotransfected with the p28 (−3,076/+129) promoter and IκB-α mutant or its control vector at a 1:1 molar ratio. Cells were treated by IFN-γ, LPS, and LPS plus IFN-γ for 7 h. Luciferase activity was measured from cell lysates. (D) The sequence of EMSA probe with the #1 predicted NF-κB–RE underlined. (E) Nuclear extracts were isolated from RAW264.7 cells after IFN-γ or LPS stimulation for 2 h. EMSA was performed with 17 μg of nuclear extract for each sample and a double-stranded oligonucleotide probe containing the -3,068/−3,026 region of the mouse p28 promoter. (F) A "supershift" EMSA was performed with the same probe and nuclear extracts from LPS-treated RAW264.7 cells. Three NF-κB–related antibodies and their control rabbit IgG were used (2 μg/lane).

demonstrates the crucial importance of NF-κB in the activation of p28 transcription.

To further explore the role of NF-κB #1 at 3,051/−3,042 in the LPS response, we performed electrophoretic mobility shift assay (EMSA) and supershift analysis using as probe the −3,068/−3,026 sequence (Fig. 6 D). As shown in Fig. 6 E, LPS treatment induced strong nuclear binding to the probe (Fig. 6 E, lane 4), which was markedly shifted by the anti–NF-κB c-Rel antibody (Fig. 6 F, lane 10) and slightly shifted by anti–NF-κB p65 antibody (Fig. 6 F, lane 8), demonstrating direct physical interaction of NF-κB c-Rel, and to a lesser extent of p65, with this NF-κB element.

Together, these results indicate that NF-κB #1 is important for p28's LPS response, but there are additional response elements localized between −1,222 to −835 of the p28 promoter.

IFN-γ/IRF-1 regulate p28 gene transcription through a specific site in the p28 promoter

To elucidate the molecular basis of IFN-γ and IRF-1–mediated transcriptional induction of p28, we localized the functional IFN-γ/IRF-1 response element in the p28 promoter region. We introduced several 5′ deletion constructs of the p28 promoter between −739 and −40, and transfected these deletion constructs (linked to a luciferase reporter gene) into the mouse macrophage cell line RAW264.7 cells activated with IFN-γ. As shown in Fig. 7 A, the response to IFN-γ was similar among all 5′ deletion constructs except the minimal construct −40, which lost substantially its ability to be stimulated by IFN-γ, indicating that a major IFN-γ "response element" (IFN-γ–RE) in the p28 promoter was likely located between −140 and −40. To determine if this region was critical for the IRF-1 response we compared the ability of the −140 and −40 constructs to be activated by exogenously introduced IRF-1. The response to IRF-1 and to IFN-γ was completely lost in the −40 construct (unpublished data), further indicating that IFN-γ–RE and the IRF-1–RE are both placed in the same region of the p28 promoter (between −140 and −40).

To more precisely localize the IFN-γ/IRF-1–RE, a computer-assisted scanning was performed, and the sequence GAAAGTGAAA located at −57 to −48 matched perfectly

with the consensus IRF-RE (reference 40; Fig. 7 B). To confirm the functional significance of this putative IRF-RE we introduced base substitutions into this site in the backbone of the full-length *p28* promoter (−739 to +129; Fig. 7 C). The WT and IRF-RE mutant promoter constructs were transiently transfected into RAW264.7 cells followed by stimulation with IFN-γ. The response of the mutant promoter to IFN-γ was completely lost compared with the response of the WT promoter (Fig. 7 C), so was the IRF-1 response (Fig. 7 D), indicating that the putative IRF-RE is indeed critical for both IFN-γ and IRF-1 responses of the p28 promoter.

Thus, it appeared that NF-κB c-Rel primarily mediates LPS-induced p28 transcription, whereas IRF-1 principally drives IFN-γ–induced p28 expression. To determine if c-Rel and IRF-1 act synergistically on p28 transcription, we cotransfected these two factors into RAW2164.7 cells with the p28 promoter. Fig. 7 E shows that IRF-1 and c-Rel each alone could activate p28 gene transcription two- to threefold. When combined, however, they induced p28 transcription more than sevenfold, demonstrating a synergistic effect by these two transcription factors.

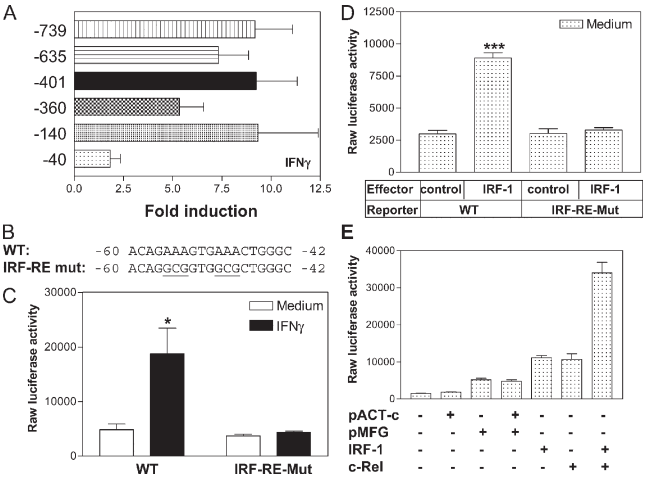

Figure 7. IFN-γ–activated p28 promoter through IRF-RE. (A) The −739/+129 and various 5′ truncation constructs of the mouse p28 promoter-luciferase reporter were transiently transfected into RAW264.7 cells by electroporation, and the transfected cells were treated with IFN-γ for 7 h. IFN-γ–induced luciferase activity from each construct was measured from cell lysates and normalized to the activity obtained with the untreated condition as relative activities (fold induction). Results shown are mean plus SE of five independent experiments. (B) Sequence of the WT mouse *p28* promoter region containing the IRF-RE, and that of the IRF-RE mutant with specific base substitutions underlined. (C) RAW264.7 cells were transiently transfected with WT or the IRF-RE mutant *p28* promoter. The transfected cells were treated with 10 ng/ml IFN-γ for 7 h. Luciferase activity was measured from the cell lyses. Data represent the mean ± SE from six independent experiments. *, $P < 0.05$, comparing IFN-γ–treated and untreated conditions. (D) RAW264.7 cells were transiently transfected with WT or the IRF-RE mutant *p28* promoter together with the IRF-1 expression vector or its control vector at a 1:1 molar ratio of reporter/effector. Cells were not further stimulated before luciferase activity measurement. Data represent the mean ± SE from six independent experiments. (E) RAW264.7 cells were transiently transfected with WT *p28* promoter together with the IRF-1 and c-Rel expression vectors or their respective control vectors pACT-C and pMFG (reference 36). Cells were not further stimulated before luciferase activity measurement. Data represent the mean ± SE from three independent experiments. ***, $P < 0.001$, comparing IRF-1–effected and control vector–transfected conditions.

IRF-1 binds to the IRF-RE of p28 promoter both in vitro and in vivo

To determine if the transcriptional activation of the *p28* promoter by IRF-1 was a direct event or via an indirect mechanism, we performed EMSA using nuclear extracts isolated from RAW264.7 cells stimulated with IFN-γ or LPS. As shown in Fig. 8 A, there was one major IFN-γ–induced nuclear DNA–binding complex formed with an oligonucleotide containing the WT *p28* promoter's IRF-RE (Fig. 8 A, lane 3, bracket). This complex was not induced by LPS (Fig. 8 A, lane 4). The binding activity was abolished with the IRF-1 mutant probe (Fig. 8 A, lanes 6–8). To further confirm the binding specificity, a competition EMSA was performed with the cold probe and various competitors. The cold probe and a separate IRF-1–RE consensus probe totally blocked the binding (Fig. 8 B, lanes 11 and 13, respectively), whereas the mutant cold probe and the consensus IRF-1–RE mutant probes could not compete at all (Fig. 8 B, lanes 12 and 14, respectively), nor could an unrelated probe (lane 15). Furthermore, a "supershift" experiment (Fig. 8 C) demonstrated that this complex indeed contained predominantly IRF-1 as an anti–IRF-1 Ab was able to strongly retard its mobility (Fig. 8 C, lane 18) but not by antibodies directed toward other members of the IRF family, namely IRF-3 and IRF-7 (Fig. 8 C, lanes 19–20). To determine if this binding activity also exists in primary macrophages we isolated nuclear extracts from WT and IRF-$1^{-/-}$ mice and performed EMSA with the same WT probe. IFN-γ induced a highly discrete binding in WT macrophages (Fig. 8 D, lane 23, arrow) but the inducible binding was completely absent under this condition in nuclear extracts isolated from IRF-$1^{-/-}$ cells (Fig. 8 D, lane 26). Interestingly, LPS treatment also induced this binding activity (Fig. 8 D, lane 24) in an IRF-1–dependent manner (Fig. 8 D, lane 27). Note that as we reported previously, in mouse peritoneal macrophages, unlike in RAW264.7 cells, both IFN-γ and LPS can induce IRF-1 expression (36). These results demonstrate that IRF-1 specifically and directly interacts with the IRF-RE in the *p28* promoter in vitro.

To determine if the interaction between IRF-1 and the p28 promoter occurs in vivo, chromatin immunoprecipitation (ChIP) assay was performed in mouse peritoneal macrophages. Fig. 8 E illustrates the region of the p28 promoter containing the IRF-RE that was examined in the ChIP assay. Fig. 8 F demonstrates that both IFN-γ and LPS induced strong binding of IRF-1 to this region of the p28 promoter detected specifically by the anti–IRF-1 antibody (Fig. 8 F, lanes 3–4) but not by the control IgG (Fig. 8 F, lanes 6–7).

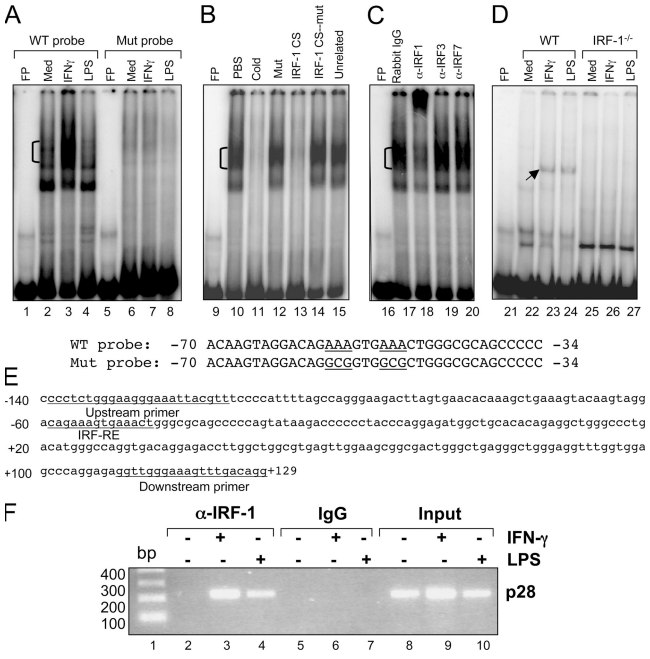

Figure 8. IRF-1 binds to *p28* promoter in vitro and in vivo. (A) Nuclear extracts were isolated from RAW264.7 cells after IFN-γ or LPS stimulation for 4 h. EMSA was performed with 10 μg of nuclear extract for each sample and a double-stranded oligonucleotide probe containing the −70/−34 region of the WT or mutant mouse p28 promoter (sequence given with the critical IRF-RE and the mutations underlined). (B) Competitive EMSA was performed with the −70/−34 probe and various competitors as indicated. (C) A supershift EMSA was performed with the −70/−34 probe and nuclear extracts from ether IFN-γ–treated RAW264.7 cells. Three IRF-related antibodies and their control rabbit IgG were used (2 μg/lane). (D) Nuclear extracts were isolated from thioglycolate-elicited primary mouse peritoneal macrophages from WT and IRF-1$^{-/-}$ mice after IFN-γ or LPS stimulation for 4 h. EMSA was performed with 10 μg of nuclear extract for each sample and a double-stranded oligonucleotide probe containing the −70/−34 region of the WT mouse p28 promoter. (E) Sequence of mouse *p28* promoter containing IRF-RE. The sequences of the pair of PCR primers used to perform ChIP are underlined as well as the IRF-RE. (F) ChIP analysis was performed in WT mouse peritoneal macrophages. The amplified mouse genomic fragment derived from the endogenous *p28* promoter encompassing the IRF-RE is indicated. The control antibody was an isotype-matched IgG. Input DNAs were used as controls.

DISCUSSION

IL-27 is the latest addition to the IL-12 family of heterodimeric cytokines composed also of IL-12 and IL-23. Members of this family share ligand and receptor subunits and play somewhat overlapping roles in innate and adaptive immune responses. These three cytokines are not entirely redundant, as they may preferentially activate naive or memory T cells, induce discrete T cell cytokine profiles, contribute to distinct stages of host immune responses to infectious agents, and differentially promote autoimmunity (41). Further elucidation of the unique functions of the IL-12 family members may lead to better understanding of T and NK cell immunobiology and improved immunodiagnostics and therapies. The physiological importance of IL- 27 compelled us to initiate this study to investigate the molecular mechanisms involved in the regulation of the production of its constituent p28 gene by macrophages.

In this study, we made several findings that were partly expected and partly surprising. First, unlike its closest relative IL-12 p35, which is not secreted alone by activated macrophages, IFN-γ alone can stimulate p28 synthesis and secretion (Fig. 1 A). However, p28, similarly to p35 or p19, is not efficiently secreted by the cells if the second chain EBI3 (or p40 for the other two cytokines) is not expressed. Although it has been reported that mouse p28 may be secreted at low levels by transfected cells, this is supposed to be very inefficient. Thus, it is possible that all or most of p28 that is present is associated with EBI3. The situation is complicated by the fact that, unlike IL-12 or IL-23, the IL-27 heterodimer is not covalently linked. Also, although the manufacturer of the p28 ELISA system indicates a cross-reactivity of 7% with IL-27, the antigen used in immunization was a hyperkine single chain molecule that may react differently with the antibody than the native protein. Thus, it is possible that the physiological heterodimer is recognized in the ELISA assay.

Again, like p35, IFN-γ and LPS have a synergism in the induction of p28 expression (Fig. 1 B). LPS stimulated much greater levels of p28 production than IFN-γ with a faster kinetics than IFN-γ (Fig. 1 B). This suggests that IFN-γ–induced IL-27 production may be a secondary event to microbial infection. Furthermore, LPS-induced p28 production, like IL-12 (42), is totally dependent on MyD88 (Fig. 2, B and C). Unlike p35, NF-κB c-Rel is partially dispensable for p28 production (Fig. 2, D and E). It would be interesting and necessary to examine the role of other common NF-κB components (p50 and p65) in this regard.

The reconstitution of IRF-1 expression in IRF-1–deficient BMDM, surprisingly, did not lead to restoration of IFN-γ–stimulated p28 expression (Fig. 4 B) unlike MyD88 reconstitution, which did fully restore LPS-induced p28 expression (Fig. 4 A), considering the transduction efficiency. Germline gene knockouts could potentially affect other genes developmentally, temporally, and spacially rather than the one targeted. In other words, we speculate that it is possible when a gene is eliminated in the germline, other genes that are dependent on this gene can be affected very early. Thus, reconstitution in a late stage of the life cycle can't rescue all the functions and activities of the targeted gene throughout the entire life of the animal. IRF-1 and p28 may fall into this kind of relationship.

Our previous study demonstrated that IRF-1 is required for IL-12 p35 gene transcription in macrophages in response to IFN-γ and partially to LPS (37). The present study revealed similar requirements of IRF-1 for IFN-γ– and LPS-stimulated p28 gene expression (Fig. 3). That introducing IRF-1 expression into primary macrophages could induce p28 expression (Fig. 3 D) indicates that IRF-1 plays an important role in the transcriptional activation of p28.

This notion is strongly buttressed with data showing that both IFN-γ– and IRF-1–stimulated p28 transcription maps to the same IRF-RE located at −57/−48 of the p28 promoter (Fig. 7, C and D) and that IFN-γ–induced endogenous IRF-1 binds to the same site both in vitro and in vivo (Fig. 8).

After the establishment that p28 expression was regulated primarily at the level of transcription (Fig. 5 A), we cloned the mouse p28 gene promoter and identified five TISs, of which one is predominantly used in macrophages (Fig. 5 D). It is possible that the other four TISs are used in different cell types or under different stimulatory conditions according to the physiological cues and needs. Our initial p28 promoter construct containing the region between −940 and +129 was rather robust in terms of its response to IFN-γ stimulation, but highly unresponsive to LPS stimulation. In the process of determining p28's TIS, we identified a transcriptional "pausing" site between +129 and +289, meaning that there is a constitutive level of transcription independent of stimulation that stops in this region unless the cell receives stimulation by LPS or IFN-γ. This observation prompted us to extend the −739/+129 p28 promoter construct further downstream to include the region between +129 and +289 with the rationale that this additional region may contain an LPS-response element. However, the more extended construct remained unresponsive to LPS (unpublished data). We then reasoned that the LPS response element may be located upstream of −940. Indeed, extending the promoter further upstream revealed at least two critical regions for LPS response, one located at −3,051/−3,042 that interacts strongly with LPS-induced c-Rel (Fig. 6), and the other located between −1,222 and −940 that remains to be fully characterized. In addition, we demonstrated that IRF-1 and c-Rel can transactivate the p28 promoter in a synergistic manner (Fig. 7 E), representing the likely combined effects of IFN-γ and LPS on p28 transcription.

In summary, we report the first study of the molecular regulatory mechanisms involved in the transcription of IL-27 p28 gene in IFN-γ– and LPS-activated macrophages. We identified the TIS within the p28 gene promoter and determined the relative roles that MyD88, c-Rel, and IRF-1 play in the regulation of p28 gene expression, and the molecular mechanisms whereby IRF-1 and NF-κB contribute critically to IFN-γ and LPS-induced p28 expression. This study will serve to lay an important foundation for further exploration of IL-27's multifaceted immunobiology in health and diseases.

MATERIALS AND METHODS

Mice. Female IRF-1$^{-/-}$ mice and their control, C57BL/6J mice (~6–8 wk old), were obtained from The Jackson Laboratories. c-Rel$^{-/-}$ mice were supplied by H.-C. Liou (Weill Medical College of Cornell University, New York, NY). A. Ding and E. Falck-Pedersen (Weill Medical College of Cornell University, New York, NY) provided the MyD88$^{-/-}$ mice originated from S. Akira (Osaka University, Osaka, Japan). All mice were housed in cages with filter tops in a laminar flow hood and fed food and acid water ad libitum at Weill Medical college of Cornell University Animal Facilities in accordance with the principles of Animal Care of the National Institutes of Health. These studies were reviewed and approved by the institutional Animal Care and Use Committee of Weill Medical College of Cornell University.

Cells. The mouse macrophage cell line RAW264.7 (RAW cells hereafter) was obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml of penicillin and streptomycin, and 10% FBS (Hyclone; endotoxin <1 ng/ml). Mouse peritoneal exudate macrophages were obtained by lavage 4 d after injection of sterile 3% thioglycolate broth (1 ml i.p.). Cells were washed and resuspended in RPMI containing 10% FCS and standard supplements. Macrophages were plated in 24-well tissue culture dishes (0.5×10^6 cells/well). After 2-h incubation to allow for adherence of macrophages, monolayers were washed three times to remove nonadherent cells and incubated with RPMI containing 10% FCS and standard supplements. The next day, 10 ng/ml IFN-γ and 1 μg/ml LPS were added at different time points.

Mouse BMDM were generated from bone marrow stem cells obtained from the femurs of the mice. After lysis of the red blood cells, 2×10^6 of bone marrow stem cells were inoculated in 60-mm Petri dishes with complete DMEM culture medium containing 10% FCS, 20% L-medium, and standard supplements. After 7-d culture, the fully differentiated and matured BMDM were treated with 10 ng/ml IFN-γ and 1 μg/ml LPS for 5 h for RNA extraction.

Plasmids. Mouse p28 promoter that extended from –3076 to +129 was amplified by PCR with genomic DNA extracted from the spleen of WT C57BL/6 mice (5′ primer: TTGGCACTGACATCACGGAACCTA; 3′ primer: CCTGTCAAACTTTCCCAACC). The PCR product was cloned into the PCR2.1 cloning vector (Invitrogen). After sequence verification the insert in PCR2.1 was excised with XhoI and HindIII and cloned into pGL2-basic luciferase vector (Promega). The IRF-1 and NF-κB mutant p28 promoter constructs were generated by site-directed mutagenesis according to manufacturer's protocol (Stratagene). Expression vector pAct-1 (IRF-1) and control pAct-C were provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). C-Rel and IκB-α mutant vectors were previously described (36). MyD88/GFP and control pMSCV-GFP retrovirus vectors were provided by A. Ding. For cloning IRF-1 lentivirus vector, the full length of mouse IRF-1 cDNA was released by EcoRI from the IRF-1/adenovirus vector (36) and blunted by Klenow. Dephosphorylated blunt-end IRF-1 cDNA was cloned into MA-1 vector, which was blunted by Klenow after cut by SmaI and SalI and then by sequence verification. The empty MA-1 lentivirus vector and its package vectors (VSV-G, pMDLg/p RRE, and pRSV-REV plasmids) were provided by S. Rivella (Weill Medical College of Cornell University, New York, NY). All plasmid DNA for transfection were prepared with Endo-free Maxi-Prep kits (QIAGEN).

Reagents. Antibodies for IRFs and NF-κB components used in this study were purchased from Santa Cruz Biotechnology, Inc. Recombinant mouse IFN-γ was purchased from Genzyme. LPS from *Escherichia coli* 0217:B8 was purchased from Sigma-Aldrich.

Retroviral and lentiviral packaging and transduction. GP2-293 packaging cells (CLONTECH Laboratories, Inc.) at ~50–70% confluency in 100-mm culture plates were transfected with 5 μg each of plasmids encoding a protein (pVSV-G; CLONTECH Laboratories, Inc.) and MyD88, using FuGENE 6 (Roche). 2 d after transfection, supernatants were cleared by centrifugation at 1,000 *g* for 10 min and were pelleted at 50,000 *g* for 90 min. Pelleted viruses were resuspended overnight at 4°C in 100 μl of 50 mM Tris-HCl, pH 7.8, 130 mM NaCl, and 1 mM EDTA. Bone marrow cells were infected with retroviral particles on day 2 and again on day 4 during their maturation in the presence of 20% L-cell medium. On day 7, mature macrophages were incubated for 5 h with fresh medium plus IFN-γ, LPS, and IFN-γ plus LPS after which RNA was isolated for further analysis.

For lentivirus generation, HEK293TN cells (System Biosciences) at 50% confluency were transfected with 3 μg ENV plasmid (VSV-G), 5 μg pMDLg/p RRE plasmid, 2.5 μg pRSV-REV plasmid, and 10 μg IRF-1/MA-1 expression plasmid or 10 μg of control GFP plasmid using FuGENE 6. Subsequent steps were identical to those for the preparation of the retrovirus described in the previous paragraph.

RT-PCR. RT-PCR reactions were performed under standard conditions (39). The following primers were used for PCR amplification of the mouse p28 cDNA: sense, CTCTGCTTCCTCGCTACCAC; antisense, GGGGCAGCTTCTTTTCTTCT; mouse IRF-1 sense, CAGAGGAAAGAGAGAAAGTCC; antisense, CACACGGTGACAGTGCTGG; mouse HPRT sense, GTTGGATACAGGCCAGACTTTGTTG; antisense, GAGGGTAGGCTGGCCTATGGCT.

qPCR. To determine the level of mRNA expression by qPCR, we used a modified protocol from Rajeevan et al. (43). In brief, cDNA converted from 1 μg of total RNA was diluted in a several concentrations. Diluted cDNA was mixed with a pair of primers (10 μM) derived from mouse p28 or GAPDH cDNA sequences and SYBR green PCR master mix (Applied Biosystems) in a 15-μl volume. PCR cycling was as follows: 2 min at 50°C, 10 min at 95°C for 1 cycle followed by 15 s at 95°C for 40 cycles, and 1 min at 60°C. The PCR primers used for mouse GAPDH were forward, AACTTTGGCATTGTGGAAGG, and reverse, ACACATTGGGGGTAGGAACA.

Enzyme-linked immunosorbent assays. Supernatants from mouse peritoneal macrophage cultures were harvested at 6, 12, and 24 h after IFN-γ and LPS stimulation and stored at −70°C. Mouse IL-27 p28 was detected using the Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions. Concentrations were calculated by regression analysis of a standard curve.

Defining the mouse p28 gene promoter region. We first used the mouse p28 mRNA sequence (available from GenBank/EMBL/DDBJ under accession no. AY099297) to blast the public database and identified a mouse chromosome 7 genomic contig containing the p28 gene (available from GenBank/EMBL/DDBJ under accession no. NT_039433.5). Based on the genomic sequence, the precise TIS was defined by RLM-RACE (see the following section). Thus, the promoter region of p28 gene is defined as sequences upstream of the TIS.

RLM-RACE. RLM-RACE was used to determine the TISs of mouse p28 gene using an assay kit (Invitrogen). In brief, 5 μg of total RNA treated with IFN-γ or LPS at different time points were dephosphorylated with calf intestinal phosphatase. The full-length capped mRNA was then treated with tobacco acid pyrophosphatase to remove the 5′ 7-methyl guanine cap of intact, mature mRNA molecules. RNA molecules that had 5′ phosphate groups, including degraded or unprocessed mRNAs lacking a 5′ cap, structural RNAs, and traces of contaminating genomic DNA, were dephosphorylated by calf intestinal phosphatase treatment and were therefore not ligated to the adaptor primer sequence. The decapped mRNA was ligated with a 44-base GeneRacer RNA oligo (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) using T4 RNA ligase. The ligated mRNA was reverse transcribed using SuperScript III RT and oligo dT primer to create RACE-ready first-strand cDNA with known priming sites at the 5′ ends. The 5′ ends of the *p28* gene transcript were amplified using two nested sense primers corresponding to the RNA oligo sequence and two nested antisense primers specific to p28 mRNA (outer, 5′-AGCATGGCAGGGAAGGGCCGAAGTGT-3′; inner, 5′-TCCCTGCGCAGCTCTTGAAGGCTCA-3′). PCR conditions were at 94°C for 2 min for 1 cycle, 94°C for 30 s, 66°C for 30 s, 68°C for 1 min for 20 cycles, and 68°C for 10 min. The PCR product was size fractionated by 1.2% agarose gel–electrophoresis. Two bands of ∼350 and 125 bp were excised from the gel and purified. The purified PCR products were cloned and sequenced. The transcription start sites of the p28 gene were defined by aligning the 5′ RLM-RACE sequences with the *p28* gene sequence.

Primary transcript measurement. To determine the primary transcription rate of p28 gene induced by IFN-γ, cDNA were synthesized with random primers using 1 μg of total RNA generated from IFN-γ–treated mouse macrophages. The region flanking intron1 and exon2 was amplified by PCR. The primers for primary transcript are as follows: p28 intron1 (sense), AGTTATGTAGGCTGGGCACTGGAA; p28 exon2 (antisense): ACGACTGCAAGATTGGAGCACTTG. PCR amply conditions were 94°C for 4 min for 1 cycle, 94°C for 15 s, 60°C for 30 s, 72°C for 30 s for 30 cycles, and 72°C for 7 min. PCR products were run on 1.2% agarose gel.

Nuclear extract preparation. Nuclear extracts for Western blot and EMSA were prepared according to the methods of Schreiber et al. (44).

Adenoviral vectors and their propagation. The construction and propagation of LacZ- and IRF-1–expressing adenovirus were described elsewhere (36, 37).

Transfection assay. Transient transfections were performed by electroporation as previously described (45).

EMSA. EMSA and supershifts were performed as described previously (46).

ChIP assay. The ChIP procedure was performed using an assay kit following the manufacturer's instructions (Upstate Biotechnology) and as previously described (36). The input and immunoprecipitated DNA were amplified by PCR using primers encompassing the IRF-1–RE in the mouse p28 promoter (5′ primer, CCCTCTGGGAAGGGAAATTACGTT; 3′ primer, CCTGTCAAACTTTCCCAACC). The samples were amplified for 30 cycles and analyzed by electrophoresis on a 1.2% agarose gel.

Statistical analysis. Student *t* test was performed wherever applicable. Standard deviation of the mean is shown unless otherwise indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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