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

Jianguo Liu, Xiuqin Guan, and Xiaojing Ma

Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021

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 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.

The Journal of Experimental Medicine

ARTICLE

JEM © The Rockefeller University Press $15.00
Vol. 204, No. 1, January 22, 2007 141–152 www.jem.org/cgi/doi/10.1084/jem.20061440

IL-27, a novel IL-12 family cytokine, is a heterodimeric molecule composed of 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 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.

CORRESPONDENCE
Xiaojing Ma:
xim2002@med.cornell.edu

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, S′ RNA ligase-mediated rapid amplification of cDNA end; TIS, transcription initiation site; TLR, Toll-like receptor.
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...
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 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 expression.
mRNA expression (Fig. 3 D, middle). The control LacZ adeno virus–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−/− bone marrow–derived macrophages (BMDM) by retrovirus and lentivirus transduction, respectively. MyD88−/− BMDM were transduced with retroviral vectors expressing GFP or MyD88/GFP (38). As shown 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−/− 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 suggest 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 primary macrophages was 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 sequences def ined five TISs in the p28 gene. Fig. 5 C shows the sequence of the p28 promoter with these five TISs.
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 −940 down to +129 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.
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 GAAAGTAAAA located at −57 to −48 matched perfectly with the predicted site.
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 RAW264.7 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.

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).
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 spatially 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. Lion (Weill Medical College of Cornell University, New York, NY). A. Ding and E. Falk-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/m 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 × 10⁶ 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 × 10⁶ of bone marrow stem cells were inoculated in 60-mm Petri dishes with complete DMEM medium containing 10% FCS, 20% t-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: TTTGCGACTGACATCGGAACTT; 3′ primer: CCTGTCAAACTTTCTCCACC). The PCR product was cloned into the PCR2.1 cloning vector (Invitrogen). After sequence verification the insert in PCR2.1 was excised with Xhol 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 mouse IRF-1 cDNA was released by EcoRI from the IRF-1/adenovirus vector (36) and blunt-end cloned into pLent1. Diphosphorylated blunt-end IRF-1 cDNA was cloned into MA-1 vector, which was blunt-end joined with Klenow after cut by SmaI and SalI and then by sequence verification. The empty MA-1 lentivirus vector and its package vectors (SVV-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 (CLONEtech Laboratories, Inc.) at ~50%–70% confluency in 100-mm culture plates were transfected with 5 μg each of plasmids encoding a protein (pSV-G; CLONEtech Laboratories, Inc.) and MyD88, using FuGENE 6 (Roche). 2 d after transfection, supernatants were collected 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% confluence were transfected with 3 μg ENV plasmid (VSV-G), 5 μg pMD-lpPAORE 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, CTCTGGTCTCCCTGGTACACC; antisense, GGGGACGCTTTCTCTTCTCT; mouse IRF-1 sense, CAGGGAAGAGAAGGCTC; antisense, CCCACGGTACAGTGCTGG; mouse HPRT sense, GTTGGATACAGGCCAGCTTTTGG; antisense, GAGGTTAGGGCTGCGGATAGCT.

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 95°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, AACCTTGCGCATGCGA, and reverse, ACACATGGGGTGGAGAACA.

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 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 3' ends 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 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'-CGACUGGAGACGAGGAGACUCGACAGUAGGCCAGAGAA-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'-AGCATGCGAGGGAAGGCGAAGGTG-3'; inner, 5'-TCCCTGGCAGCTCT-TGAAGGCCCTA-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), AGTTATGAGCCTGGGACTGAA; p28 exon2 (antisense); ACGACGTCGACATTGACGTGG. PCR amplification 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′ promoter, CCCCTCGAGGAGAGCTAGG; 3′ promoter, CCTGCTAACTTTCCAACC). 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.

We thank Dr. Ai-hao Ding and Dr. Dongxu Sun’s technical advice in preparation of the MyD88 retrovirus. This work was supported by a grant from the National Institutes of Health (CA100223) to X. Ma. The authors have no conflicting financial interests.

Submitted: 7 July 2006 Accepted: 6 December 2006

REFERENCES

1. Pflanz, S., J.C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hübner, T. Churakov, M. Evans, T. Vasik et al. 2002. IL-27, a heterodimeric cytokine composed of EB13 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity. 16:779–790.
2. Yoshida, H., S. Hamano, G. Senaldi, T. Covy, R. Faggioni, S. Mu, M. Xia, A.C. Wakeham, H. Nishina, J. Potter et al. 2001. WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. Immunity. 15:569–578.
3. Artis, D., L.M. Johnson, K. Joyce, C. Saris, A. Villarino, C.A. Hunter, and P. Scott. 2004. Cutting edge: early IL-4 production governs the requirement for IL-27–WSX-1 signaling in the development of protective Th1 cytokine responses following Leishmania major infection. J. Immunol. 172:4672–4675.
4. Schmidt, C., T. Giese, B. Ludwig, I. Mueller-Molaian, T. Marth, S. Zeuzem, S.C. Meuer, and A. Stallmach. 2005. Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn’s disease but not in ulcerative colitis. Inflamm. Bowel Dis. 11:16–23.
5. Honda, K., K. Nakamura, N. Matsui, M. Takahashi, Y. Kitamura, T. Mizutani, N. Harada, H. Nawata, S. Hamano, and H. Yoshida. 2005. T helper 1-inducing property of IL-27/WSX-1 signaling is required for the induction of experimental colitis. Inflamm. Bowel Dis. 11:1044–1052.
6. Li, J., B. Gran, G.X. Zhang, A. Rostami, and M. Kamoun. 2005. IL-27 subunits and its receptor (WSX-1) mRNAs are markedly up-regulated in inflammatory cells in the CNS during experimental autoimmune encephalomyelitis. J. Neurovirol. 11:323–39.

7. Goldberg, R., G. Wildbaum, Y. Zohar, G. Maor, and N. Karin. 2004. Suppression of ongoing adjuvant-induced arthritis by neutralizing the function of the p28 subunit of IL-27. J. Immunol. 173:1171–1178.

8. Miyazaki, Y., H. Inoue, M. Matsumura, K. Matsumoto, T. Nakano, M. Tsuda, S. Hamano, A. Yoshimura, and H. Yoshida. 2005. Exacerbation of experimental allergic asthma by augmented Th2 responses in WSX-1–deficient mice. J. Immunol. 175:2401–2407.

9. Owaki, T., M. Asakawa, S. Kamiya, T. Takeda, F. Fukui, J. Mizuguichi, and T. Yoshimoto. 2006. IL-27 suppresses CD28-mediated [correction of medicated] IL-2 production through suppression of cytokine signaling 3. J. Immunol. 176:2773–2780.

10. Villarino, A.V., J.S. Stumhofer, C.J. Saris, R.A. Kastelein, F.J. de Sauvage, and C.A. Hunter. 2006. IL-27 limits IL-2 production during Th1 differentiation. J. Immunol. 176:237–247.

11. Villarino, A., L. Hibbert, L. Lieberman, E. Wilson, T. Mak, H. Yoshida, R.A. Kastelein, C. Saris, and C.A. Hunter. 2003. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. Immunity. 19:649–655.

12. Hamano, S., K. Himeno, Y. Miyazaki, K. Iishi, A. Yamanaka, A. Takeda, M. Zhang, H. Hisaeda, T.W. Mak, A. Yoshimura, and H. Yoshida. 2003. WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production. Immunity. 19:657–667.

13. Yamanaka, A., S. Hamano, Y. Miyazaki, K. Iishi, A. Takeda, T.W. Mak, K. Himeno, A. Yoshimura, and H. Yoshida. 2004. Hyperproduction of proinflammatory cytokines by WSX-1–deficient NKT cells in concanavalin A–induced hepatitis. J. Immunol. 172:3590–3596.

14. Stumhofer, J., A. Laurence, E.H. Wilson, E. Huang, C.M. Tato, L.M. Johnson, A.V. Villarino, Q. Huang, A. Yoshimura, D. Sehy, et al. 2006. Interleukin 27 negatively regulates the development of interleukin 17–producing T helper cells during chronic inflammation of the central nervous system. Nat. Immunol. 7:937–945.

15. Batten, M., J. Li, S. Yi, N.M. Kljavin, D.M. Danilenko, S. Lucas, J. Lee, F.J. de Sauvage, and N. Ghildari. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17–producing T cells. Nat. Immunol. 7:929–936.

16. Yoshimura, T., A. Takeda, S. Hamano, Y. Miyazaki, I. Kinjyo, T. Ishibashi, A. Yoshimura, and H. Yoshida. 2006. Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23–induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism. J. Immunol. 177:5377–5385.

17. Hisada, M., S. Kamiya, K. Fujita, M.L. Belladonna, T. Aoki, Y. Koyanagi, J. Mizuguichi, and T. Yoshimoto. 2004. Potent antitumor activity of interleukin-27. Cancer Res. 64:1152–1156.

18. Chiyon, M., O. Shimozato, T. Iizasa, T. Fujisawa, and M. Tagawa. 2004. Antitumor effects produced by transduction of dendritic cells–derived heterodimeric cytokine genes in murine colon carcinoma cells. Anticancer Res. 24:3763–3767.

19. Chiyon, M., O. Shimozato, L. Yu, K. Kawayama, T. Iizasa, T. Fujisawa, and M. Tagawa. 2005. Expression of IL-27 in murine carcinoma cells produces antitumor effects and induces protective immunity in inoculated host animals. Int. J. Cancer. 115:437–442.

20. Saucedo, R., J.K. Staufler, E. Lincoln, T.C. Back, J.A. Hilton, C. Hahn, K. Shafer-Weaver, A. Malyguine, R. Kastelein, and J.M. Wiggington. 2004. IL-27 mediates complete regression of orthotopic primary and metastatic murine neuroblastoma tumors: role for CD8+ T cells. J. Immunol. 173:7170–7182.

21. Shinizu, M., M. Shimamura, T. Owaki, M. Asakawa, K. Fujita, M. Kudo, Y. Iwakura, T. Takeda, A.D. Luster, J. Mizuguichi, and T. Yoshimoto. 2006. Antiangiogenic and antitumor activities of IL-27. J. Immunol. 176:7317–7324.

22. Takeda, K., T. Kasibo, and S. Akira. 2003. Toll-like receptors. Annu. Rev. Immunol. 21:335–376.
regulators of cell growth and the interferon system. Mol. Cell. Biol. 13:4531–4538.

41. Beadling, C., and M.K. Slika. 2006. Regulation of innate and adaptive immune responses by the related cytokines IL-12, IL-23, and IL-27. Arch. Immunol. Ther. Exp. (Warsz.). 54:15–24.

42. Nomura, F., S. Akashi, Y. Sakao, S. Sato, T. Kawai, M. Matsumoto, K. Nakamichi, M. Kimoto, K. Miyake, K. Takeda, and S. Akira. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J. Immunol. 164:3476–3479.

43. Rajeevan, M.S., D.G. Ramamukkhaarachchi, S.D. Vernon, and E.R. Unger. 2001. Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. Methods. 25:443–451.

44. Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with ‘mini-extracts’, prepared from a small number of cells. Nucleic Acids Res. 17:6419.

45. Ma, X., J.M. Chow, G. Gri, G. Carra, F. Gerba, S.F. Wolf, R. Dzialo, and G. Trinchieri. 1996. The interleukin 12 p40 gene promoter is primed by interferon γ in monocytic cells. J. Exp. Med. 183:147–157.

46. Ma, X., M. Neurath, G. Gri, and G. Trinchieri. 1997. Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. J. Biol. Chem. 272:10389–10395.