Lipopolysaccharide-Mediated IL-10 Transcriptional Regulation Requires Sequential Induction of Type I IFNs and IL-27 in Macrophages

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*J Immunol* published online 1 November 2010

http://www.jimmunol.org/content/early/2010/11/01/jimmunol.1002041
Lipopolysaccharide-Mediated IL-10 Transcriptional Regulation Requires Sequential Induction of Type I IFNs and IL-27 in Macrophages

Shankar Subramanian Iyer,* Amir Ali Ghaifari,† and Genhong Cheng†,‡

IL-10 is a potent anti-inflammatory molecule that regulates excessive production of inflammatory cytokines during an infection or tissue damage. Dysregulation of IL-10 is associated with a number of autoimmune diseases, and so, understanding the mechanisms by which IL-10 gene expression is regulated remains an important area of study. Macrophages represent a major source of IL-10, which is generated in response to TLR signaling as a feedback mechanism to curtail inflammatory response. In this study, we identify a signaling pathway in murine bone marrow-derived macrophages in which activation of TLR4 by LPS induces the expression of IL-10 through the sequential induction of type I IFNs followed by induction and signaling through IL-27. We demonstrate that IL-27 signaling is required for robust IL-10 induction by LPS and type I IFNs. IL-27 leads directly to transcription of IL-10 through the activation of two required transcription factors, STAT1 and STAT3, which are recruited to the IL-10 promoter. Finally, through systematic functional promoter-reporter analysis, we identify three cis elements within the proximal IL-10 promoter that play an important role in regulating transcription of IL-10 in response to IL-27. The Journal of Immunology, 2010, 185: 000–000.

Immune cells respond to bacterial or viral infection by the rapid activation of proinflammatory cytokines that serve to initiate host defense against microbial invasion. However, excess proinflammatory cytokines give rise to systemic metabolic and hemodynamic disturbances that are harmful to the host. To avert these deleterious effects, IL-10 is produced by macrophages as a negative-feedback mechanism to dampen uncontrolled production of inflammatory cytokines and excessive inflammation during infection. IL-10 is a potent anti-inflammatory cytokine with a broad effect on both innate and adaptive immune systems.

Induction of innate immunity is mediated by diverse families of pattern recognition receptors that recognize microbial components termed pathogen-associated molecular patterns, which can be viewed as a molecular signature of the invading pathogens. TLRs are a major family of pattern recognition receptors that are mainly expressed by cells of the innate immune system (1, 2). TLRs can initiate distinct innate immune responses through recruitment of different MyD88 adaptor family members, primarily MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (3–4). Currently, 11 TLRs have been cloned in mammals, and each receptor is involved in the recognition of a unique set of pathogen-associated molecular patterns (e.g., TLR4 recognizes LPS) (2, 5, 6). Although it is well established that IL-10 is induced in innate immune cells in response to TLR agonists like LPS, the molecular events responsible for upregulation of IL-10 remain to be elucidated. In addition, because LPS induction of IL-10 requires signaling through both feed-forward and feedback loops that may mitigate aspects of IL-10 regulation, it is difficult to identify transcription factors and cis elements within the IL-10 promoter.

We and others have identified two major signaling pathways mediated by TLR4 activation: the MyD88-dependent activation of NF-κB that results in the induction of inflammatory genes, such as TNF, IL-6, and IL-1β, and TRIF-dependent pathways involving the induction of type I IFNs and, subsequently, secondary-response genes induced by IFN-β in an autocrine/paracrine manner. The TRIF adaptor molecule has been shown to be indispensable for TLR4-mediated IL-10 activation (4, 7–10).

Classically, type I IFNs (IFN-β/α) bind to a cognate heterodimeric IFNAR (IFN-α/βR) to activate the Jak–STAT pathway leading to expression of antiviral genes. In addition to their antiviral functions, type I IFNs are capable of exerting immunomodulatory effects on both innate and adaptive immune cells, in large part by inducing expression of IL-10 (11–14). Previously, we have demonstrated that type I IFN signaling is required for TLR-mediated induction of IL-10 (11, 15). In addition, we have demonstrated that type I IFNs are required for TLR-mediated induction of another anti-inflammatory cytokine, IL-27, in a dose-dependent manner (15).

IL-27 functions as a heterodimer composed of p28 and EBV-induced gene 3 (Ebi3), which have homologies to IL-12p35 and p40, respectively. The IL-27R complex consists of the unique subunit IL-27R (also referred to as TCCR and WSX-1) and the gp130 chain of IL-6R, which then activate transcription factors STAT1 and STAT3 via Jak-mediated phosphorylation (16, 17). IL-27 is produced by innate immune cells and has potent immunosuppressive effects on T cell immunity, including the inhibition of Th1 and Th1 differentiation, as well as in several infection models (15, 18–22). In addition, IL-27R−/− deficient mice develop excessive tissue inflammation in the context of infection or in autoimmune conditions (18, 23, 24). Importantly, although the
underlying molecular mechanisms of IL-27–mediated immune suppression are not well understood, a number of studies have highlighted the importance of IL-27–mediated production of IL-10 to promote an anti-inflammatory state in lymphocytes (16, 25, 26). To date, the role of IL-27 in the regulation of IL-10 in macrophages is less understood.

In this study, we reveal a pathway in which LPS stimulation leads to induction of IL-10 in bone marrow-derived macrophages (BMDMs) through the sequential progression of type I IFN induction followed by IL-27 activation in an autocrine/paracrine manner. Importantly, induction of IL-10 by IL-27 in BMDMs is direct, allowing us to identify three cis regulatory elements through functional characterization of the murine IL-10 promoter. IL-27–mediated IL-10 expression is STAT3 and STAT1 dependent, and we demonstrate that both transcription factors are mobilized to the IL-10 promoter in vivo upon stimulation with LPS, type I IFNs, and IL-27.

**Materials and Methods**

**Plasmids**

A ~2-kb fragment of the IL-10 promoter (~1954-644) was amplified by PCR from mouse genomic DNA and subsequently cloned into the XhoI and HindIII sites of the pcGL4.20 polylinker (Promega, Madison, WI). Promoter deletion mutants were amplified from the ~1954-644 promoter clone by PCR using an upstream primer containing a XhoI restriction site and a downstream primer containing a HindIII site. The PCR products were then inserted into the luciferase reporter pGL4.20. Substitution mutants were generated via a two-step PCR procedure using overlapping internal primers that contain a mutant sequence. All plasmids used in transient transfection assays were purified using an endotoxin-free purification system (Invitrogen Purelink, Invitrogen, Carlsbad, CA). Substitution mutants are presented in Fig. 5A.

**BMDM preparation and mice**

Wild-type and IL-27R knockout (TCCR/WSX1−/−), STAT1+/+ (STAT1flox/flox) BMDMs were prepared and mice backcrossed with C57BL/6 for six generations. The following Abs were used at 1:1000 concentrations: anti-Sp1, anti-STAT2 (Cell Signaling Technology), anti–phospho-STAT3 (Cell Signaling Technology), anti-STAT1 (Cell Signaling Technology), phospho-STAT1 (Cell Signaling Technology), anti–phospho-STAT1 (Y701), and anti–phospho-STAT3 (Y705) (Cell Signaling Technology, Beverly, MA), and c-Maf (Santa Cruz Bio-technology).

Chromatin immunoprecipitation (ChIP) was performed by cross-linking stimulated cells with formaldehyde (1% final concentration). Cells were lysed in cell lysis buffer (5 mM PIPES [pH 7.5], 0.5% sodium deoxycholate, 1 mM EDTA, and 1% SDS) supplemented with 20 mM HCl. Chromatin was diluted to 300 μg/ml and sonicated to <150 bp (using a Diagenode Biorupter (Diagenode, Denville, NJ)) at 4°C for 1 h. Five percent of input was collected for Ab normalization. Beads were washed twice in high-salt wash buffer (50 mM HEPES [pH 7.9], 0.5 M NaCl, 1 mM EDTA, and 1% SDS) and incubated in situ with beads for 1 h. beads were washed twice in high-salt wash buffer (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 0.1% SDS, and 1% Triton X-100 supplemented with protease inhibitors (Roche Diagnostic Systems)). Sonication was performed using a Diagenode Biorupter (Diagenode, Denville, NJ) for 30 min (30 s on/off) twice to generate sheared fragments of ~500 bp. A total of 100 μg chromatin was diluted to 300 μl in dilution buffer (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 0.1% SDS, and 1% Triton X-100 supplemented with protease inhibitors) and incubated with superparamagnetic beads (Upstate Biotechnology, Lake Placid, NY) at 4°C for 1 h. Five percent of input was collected for Ab normalization. Beads were washed twice in high-salt wash buffer (50 mM HEPES [pH 7.9], 0.5 M NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 0.1% deoxycholate) and twice with TE buffer for 10 min room temperature. Beads were resuspended in 300 μl elution buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, and 1% SDS supplemented with 20 μg proteinase K (Invitrogen) for 1 h. DNA was PCR purified (Invitrogen PuriLink, Invitrogen), and immunoprecipitated DNA fragments were sequenced via qPCR using the following primers: IL-10 proximal promoter forward: 5′-GGACCAAGAAGGACTG-3′ and reverse: 5′-GCGAATATTTGCAAGAA-3′; IL-10 promoter forward: 5′-ACAATTCGACCAACAAC-3′ and reverse: 5′-CTTGGTGGTATTCCTCTCG-3′; and IL-10 promoter forward: 5′-CGCTCTTCTGCTGACTGCTCCT-3′ and reverse: 5′-CAGCTGGTTTCTGCTCTCCT-3′.

**Cell lines, reagents, and transfection**

The RAW264.7 murine macrophage cell line (American Type Culture Collection, Manassas, VA) was maintained in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin (complete DMEM). LPS (Escherichia coli, strain O55:B5, Sigma-Aldrich, St. Louis, MO), murine IFN-α (catalog number 12110-9, PBL InterferonSource, Piscataway, NJ), and IL-27 (catalog number 1799-ML, R&D Systems, Minneapolis, MN) were used at the concentrations described in the figures. Murine IFN-γ on ice at 4°C for 1 h. DNA was resuspended in 300 μl elution buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, and 1% SDS supplemented with 20 μg proteinase K (Invitrogen) for 1 h. DNA was PCR purified (Invitrogen PuriLink, Invitrogen), and immunoprecipitated DNA fragments were sequenced via qPCR using the following primers: IL-10 proximal promoter forward: 5′-GGACCAAGAAGGACTG-3′ and reverse: 5′-GCGAATATTTGCAAGAA-3′; IL-10 promoter forward: 5′-ACAATTCGACCAACAAC-3′ and reverse: 5′-CAGCTGGTTTCTGCTCTCCT-3′; and IL-10 promoter forward: 5′-CGCTCTTCTGCTGACTGCTCCT-3′ and reverse: 5′-CAGCTGGTTTCTGCTCTCCT-3′.
and reverse: 5'-ACTAAAAGTTGTATTTCC-3', which amplifies 225 bp from -228 to -4 relative to the transcription start site; and IL-10 distal promoter forward: 5'-CCCCCTCCGTGCTTG-3' and reverse: 5'-GAGGGTTACCAACACAGGG-3', which amplifies 221 bp from -1353 to -1130 relative to the transcription start site.

Results

LPS, type I IFN, and IL-27 signaling induce IL-10 expression in BMDMs

Previously, we had demonstrated LPS mediated induction of IL-10 through a TRIF-dependent mechanism that involves the production and signaling of type I IFNs (11, 28–30). In light of a role of IL-27 as an anti-inflammatory agent and a potent inducer of IL-10, we asked whether IL-27 signaling was involved in LPS-mediated IL-10 induction. To address this question, we stimulated wild-type, IFN-α-deficient, and IL-27R–deficient BMDMs with LPS and assessed IL-10 protein production over time via ELISA. As expected, LPS-mediated production of IL-10 was deficient in BMDMs lacking IFN-αR (Fig. 1A). Surprisingly, IL-27R–deficient BMDMs also exhibited significant defects in IL-10 production in response to LPS (Fig. 1A). We then confirmed IL-10 protein production in BMDMs in response to LPS, IFN-α, and IL-27 (Fig. 1B). To assess IL-10 expression at the transcriptional level, relative IL-10 mRNA production was assessed after 4 h of stimulation with LPS, IFN-α, and IL-27 by qPCR. Data are presented normalized to L32 mRNA levels (Fig. 1C). Importantly, relative to induction of proinflammatory cytokines like IL-12p40 and IL-6, IL-10 transcription in response to LPS appeared delayed, with detectable transcripts first appearing after 2 h of stimulation. In contrast, both IFN-α and IL-10 displayed faster kinetics of IL-10 transcription, with both agents able to generate detectable transcripts within 1 h of stimulation, even at low-dose concentration (data not shown).

IL-27 signaling occurs downstream of TLR4 and type I IFNs in LPS-mediated IL-10 gene expression

Previously, we had demonstrated LPS-mediated induction of IL-10 in wild-type through a TRIF-dependent mechanism that involves the production and signaling of type I IFNs (11, 28–30). In light of a role of IL-27 as an anti-inflammatory agent and a potent inducer of IL-10, we asked whether IL-27 signaling was involved in LPS-mediated IL-10 induction. To address this question, we stimulated wild-type, IFN-α-deficient, and IL-27R–deficient BMDMs with LPS and assessed IL-10 protein production over time via ELISA. As expected, LPS-mediated production of IL-10 was deficient in BMDMs lacking IFN-αR (Fig. 1A). Surprisingly, IL-27R–deficient BMDMs also exhibited significant defects in IL-10 production in response to LPS (Fig. 1A). We then confirmed IL-10 protein production in BMDMs in response to LPS, IFN-α, and IL-27 (Fig. 1B). To assess IL-10 expression at the transcriptional level, relative IL-10 mRNA production was assessed after 4 h of stimulation with LPS, IFN-α, and IL-27 by qPCR. Data are presented normalized to L32 mRNA levels (Fig. 1C). Importantly, relative to induction of proinflammatory cytokines like IL-12p40 and IL-6, IL-10 transcription in response to LPS appeared delayed, with detectable transcripts first appearing after 2 h of stimulation. In contrast, both IFN-α and IL-10 displayed faster kinetics of IL-10 transcription, with both agents able to generate detectable transcripts within 1 h of stimulation, even at low-dose concentration (data not shown).

FIGURE 1. Induction of IL-10 protein and mRNA in BMDMs. A, Total of 0.5 × 10^6 WT, IFN-αR KO, and IL-27R KO BMDMs were stimulated with LPS (50 ng/ml), and supernatant was collected at the indicated time points. IL-10 protein production was quantified via ELISA. B, Total of 0.5 × 10^6 BMDMs were unstimulated or stimulated with LPS (50 ng/ml), IFN-α (250 U/ml), or IL-27 (10 ng/ml), supernatant was collected, and IL-10 protein production was quantified via ELISA. C, Total of 0.5 × 10^6 BMDMs were unstimulated or stimulated with LPS (50 ng/ml), IFN-α (250U/ml), or IL-27 (10 ng/ml) for 4 h, RNA was harvested, and relative IL-10 transcript was detected via qPCR normalized to L32.

significantly abrogated compared with wild-type (Fig. 2C, left panel). IFN-α was unable to induce IL-10 in IFN-αR–deficient BMDMs due to lack of its receptor (Fig. 2C, middle panel). However, IL-10 was similarly induced in wild-type and IFN-αR–deficient BMDMs by rIL-27 stimulation (Fig. 2C, right panel). These data suggest that IL-27 can induce IL-10 expression independent of type I IFN signaling, whereas LPS and IFN-α cannot.

Next, we assessed the requirements for IL-27R signaling on LPS and IFN-α–mediated IL-10 gene expression. In this study, we treated previously unstimulated wild-type and IL-27R–deficient BMDMs with LPS-CM and IFN-α-CM for 3 h and monitored IL-10 expression by qPCR. Both LPS- and IFN-α–mediated induction of IL-10 were significantly decreased in IL-27R–deficient BMDMs compared with wild-type (Fig. 2B). We confirmed these results by inducing wild-type and IL-27R–deficient BMDMs with LPS, rIFN-α, and rIL-27 directly. Again, LPS and IFN-α were able to robustly induce IL-10 in IL-27R null BMDMs compared with wild-type (Fig. 2D, left and middle panels). IL-27 was unable...
to induce IL-10 expression in the IL-27R-deficient cells due to the absence of its cognate receptor (Fig. 2D, right panel). These data suggest that IL-27 signaling occurs downstream of TLR4 and type I IFN in the induction of IL-10 expression.

IL-27 induction of IL-10 is direct, whereas LPS- and IFN-α–mediated IL-10 expression require de novo protein synthesis

Our data suggest that both LPS and type I IFN induction of IL-10 occurs indirectly through subsequent signaling via IL-27. Previously, our laboratory and others have shown that LPS-mediated induction of IL-10 requires de novo synthesis and signaling by type I IFNs for robust induction of IL-10 transcripts (11). We next wanted to assess whether induction of IL-10 by IL-27 was direct or required subsequent de novo synthesis of a transcription factor or signaling molecule. To ascertain this, we pretreated wild-type BMDMs with CHX, a translation inhibitor, followed by stimulation with LPS, rIFN-α, or rIL-27. Inhibition of protein synthesis led to defects in LPS and IFN-α–mediated IL-10 induction (Fig. 2E, left and middle panels). In contrast, IL-27–induced IL-10 expression was unaffected by CHX treatment, suggesting that IL-27–mediated IL-10 transcription is direct (Fig. 2E, right panel).
LPS signaling leads to induction of both type I IFNs and IL-27p28 transcripts with induction of IL-27p28 displaying delayed kinetics of expression relative to IFN-β. Because both LPS- and IFN-α-mediated induction of IL-10 require IL-27 signaling and de novo protein synthesis, we propose that both LPS and IFN-α induce expression of IL-27 and that subsequent IL-27 signaling leads to robust IL-10 gene expression. To test this, we assessed transcription of the IL-27p28 gene in WT and IFN-αR−/− BMDMs treated with LPS-CM for 3 h. Expression of IL-27p28 by LPS-CM occurred in WT but not IFN-αR−/−-deficient BMDMs (Fig. 3B). Induction of the second subunit of IL-27, EBI3, was not robust, and no significant differences between WT and IFN-αR−/− KO were observed (data not shown). We confirmed these results by stimulating wild-type and IFN-αR−/−-deficient BMDMs directly with LPS and rIFN-α. LPS-mediated induction of both IL-27p28 and EBI3 was type I IFN dependent (Fig. 3C). IFN-α was able to induce both IL-27p28 and EBI3 in wild-type but not IFN-αR−/−-deficient cells due to loss of its receptor. Interestingly, induction of IL-27p28 by LPS was abrogated in the presence of CHX, whereas IFN-α–mediated induction of IL-27p28 was not, suggesting that type I IFNs can induce IL-27 gene expression directly, whereas LPS requires de novo protein synthesis, presumably of type I IFNs (data not shown).

LPS induction of type I IFNs, IFN-β or IFN-α, is mediated primarily through the signaling by the TRIF adaptor molecule, leading to recruitment of the enhanceosome to the IFN-β promoter, resulting in gene induction (31, 32). Subsequent signaling of IFN-β leads to formation of the ISGF3 complex, leading to induction of genes as well as type I IFN-mediated antiviral gene products, via qPCR normalized to L32. Data represent mean ± SEM. A Student’s t test was performed. *p < 0.01.

**FIGURE 3.** LPS induction of IL-10 requires sequential induction of type I IFNs and IL-27, respectively. A, Total of 0.5 × 10^6 WT BMDMs were stimulated with LPS (50 ng/ml) for the indicated time points. RNA was collected, and IFN-β, IFN-α, and IL-27p28 transcript level was detected via qPCR normalized to L32. B, Total of 0.5 × 10^6 WT were unstimulated or stimulated with LPS for 2 h. CM was collected and transferred to 0.5 × 10^6 WT or IFN-αR−/− previously serum-starved (1% FBS) BMDMs in the absence of any stimulation. Cells were incubated with the indicated CM for 3 h, RNA was collected, and IL-27p28 transcript level was detected via qPCR normalized to L32. C, Total of 0.5 × 10^6 WT or IFN-αR−/− BMDMs were unstimulated or stimulated with LPS (50 ng/ml) or IFN-α (250 U/ml) for 3 h. RNA was collected, and IL-27p28 or EBI3 transcript level was detected via qPCR normalized to L32. D, Total of 0.5 × 10^6 WT or IL-27R−/− BMDMs were unstimulated or stimulated with LPS (50 ng/ml), IFN-α (250 U/ml), or rIL-27 (10 ng/ml) for 3 h. RNA was collected, and IFN-α and IL-27p28 transcript level was detected via qPCR normalized to L32. Data represent mean ± SD from three independent experiments. Student t test was performed. *p < 0.01.
signaling that can mask functional redundancy in the regulation of gene expression via multiple signaling pathways.

We cloned a fragment of the proximal IL-10 promoter extending from nucleotide −1954 to +64 relative to the +1 transcription start site (35), inserted into a firefly luciferase reporter vector. We then transiently transfected the promoter–reporter construct into the macrophage cell line RAW 264.7 to assess promoter activity. Importantly, RAW 264.7 cells exhibit similar gene profiles to BMDMs in response to LPS (28, 29). Following transfection, cells were stimulated with LPS, IFN-α, or IL-27 for 12 h. Each stimulant was able to induce robust IL-10 promoter activity compared with the empty luciferase vector. To make meaningful comparisons among LPS, IFN-α, and IL-27, we titrated each stimulatory agent and chose activation concentrations that resulted in a similar level of relative IL-10 promoter induction, normalized to renilla luciferase activity to control for transfection efficiency (Fig. 4A).

To identify DNA sequences that are necessary for LPS-, IFN-α-, or IL-27–induced promoter activity, a series of promoter mutants that contain successive deletions from the 5′ end were inserted upstream of the luciferase reporter gene. Each mutant shared the common +1 transcription start site. We then assessed the promoter activity in response to LPS, IFN-α, and IL-27 and measured relative induction as a percentage of the full-length −1954/+64 full-length IL-10 proximal promoter. Although some deletion constructs demonstrated slight defects in basal promoter activity, all were able to be robustly induced by LPS. We identified two major regions (indicated by arrows) located between −1232/−1541 and −78/−118 that, when deleted, led to defects in LPS-mediated promoter induction (Fig. 4B). IFN-α–mediated IL-10 promoter activity required elements within the −455/−595 and −78/−118 (Fig. 4C, indicated by arrows). Our analysis of IL-10 promoter activity in response to IL-27 revealed three major cis elements that appear to be important for promoter induction: −1232/−1541, −455/−595, and −78/−118 (Fig. 4D). Thus, although it appears that LPS, IFN-α, and IL-27 exert differential effects on the IL-10 promoter, they each share a common regulatory region located between −78/−118 relative to the transcription start site that corresponds to a previously identified Sp1 binding site (30). Interestingly, there was an additional element located in a region between −118/−240 that may act as a negative regulatory element in response to IL-27 due to an increase in promoter activity when absent. With regards to this study, this putative negative regulatory element was not studied further. In addition, LPS and IL-27 share a common regulatory element located between −1232/−1541, whereas IFN-α and IL-27 appear to require a functional element located between −455/−595. These data emphasize that although LPS and IFN-α may induce IL-10 in distinct ways, they potentially share common regulatory elements within the IL-27 pathway, which they both require for robust IL-10 gene expression.

To further characterize the regulatory elements identified in our promoter-reporter analyses, we constructed 6-mer mutations across the entirety of the three regulatory regions identified in our promoter deletion analysis: −1232/−1541, −455/−595, and −78/−90 region as well as a neutral region, −745/−938, in the context of our full-length proximal promoter (−1954/+64) and assessed their function in response to LPS, IFN-α, and IL-27 using the same transfection/stimulation strategy described earlier. The sequences of four representative promoter mutations are displayed in Fig. 5A. None of the 6-mer mutant constructs displayed any significant defects in basal IL-10 promoter activity. Only the −78/−90 region, previously described as an Sp1 binding site, was universally required for IL-10 promoter activity in response to LPS, IFN-α, and IL-27 (Fig. 5B–D). No other scanning 6-mer mutations exerted any significant effects on LPS or IFN-α–mediated IL-10 induction in the context of the −1954/+64 promoter (Fig. 5B, 5C). However, we identified two cis regulatory elements located at −1324/−1319 and −510/−504 that led to significant defects in IL-27–mediated promoter activity (Fig. 5D).

**IL-27–mediated IL-10 gene induction is regulated by STAT1 and STAT3**

We next sought to identify potential transcription factors that mediate LPS-, IFN-α-, and IL-27–dependent IL-10 gene induction. A scan of the proximal IL-10 promoter using the program MatInspector to search for putative transcription factor binding sites, revealed that the −1324/−1319 and −510/−504 cis regulatory elements that mediate IL-27–dependent IL-10 promoter activity correspond to a putative STAT3 and a previously identified c-Maf binding motif (36, 37). IL-27 signaling through the heterodimeric receptor, IL-27R/gp130, is known to activate several members of the STAT transcription family, including STAT1, STAT2, STAT3, STAT4, and STAT6. To assess activation of the STAT family of transcription factors in macrophages, we stimulated BMDMs with LPS, IFN-α, and IL-27 at the indicated time points and assessed phosphorylation of STAT1, STAT2, STAT3, and STAT5 using specific phospho-
I L - 1 0 i n d u c t i o n ( F i g . 6  dependent, whereas LPS and IFN-
IL-27; however, c-Maf appears to be constitutively expressed in
shown). We also assessed induction of c-Maf by LPS, IFN-
STAT3 phosphorylation (Fig. 6
molecules. We expected that STAT1 and STAT3 could potentially
derived from the −154/−64 IL-10 promoter reporter construct. Constructs were transfected in RAW 264.7 cells and assessed for luciferase activity as described in B. Sequences of representative wild-type or substitution mutants are indicated. B, Empty pGL4.20, −154/−64 IL-10 promoter, and mutant IL-10 promoter constructs (−154/−64 backbone) were transfected into RAW 264.7 cells and left unstimulated or stimulated with LPS (1 µg/ml) (B), rIFN-α (1000 U/ml) (C), and rIL-27 (80 ng/ml) (D). Luciferase activity is displayed as percent of full-length wild-type (−154/−64) IL-10 promoter activity normalized to renilla activity representing three independent experiments. Student t test was performed. *p < 0.05.

Tyr Abs. LPS, IFN-α, and IL-27 were able to activate STAT1 and STAT3 phosphorylation (Fig. 6a), whereas LPS and IFN-α, but not IL-27, were able to activate STAT2 phosphorylation (data not shown). We also assessed induction of c-Maf by LPS, IFN-α, and IL-27; however, c-Maf appears to be constitutively expressed in BMDMs and not subject to regulation at the protein level during the time points measured (37).

We next assessed the requirement for STAT1 and STAT3 in IL-10 induction. We stimulated wild-type and STAT1-deficient BMDMs with LPS, IFN-α, and IL-27 for 3 h and assessed IL-10 induction by qPCR. IL-27-mediated induction of IL-10 had an absolute requirement for STAT1, whereas IFN-α-mediated IL-10 induction exhibited partial but significant defects (Fig. 6B). Although there was a partial decrease in IL-10 induction in BMDMs deficient in STAT1 compared with wild-type in response to LPS, the difference was not statistically significant. Next, we stimulated wild-type and macrophage-specific STAT3+/+ and STAT3−/− BMDMs were unstimulated or stimulated with LPS (50 ng/ml), IFN-α (250 U/ml), or IL-27 (10 ng/ml) for 3 h. RNA was harvested, and relative IL-10 transcript was detected via qPCR normalized to L32. Student t test was performed. *p < 1 × 10−5. C, Total of 0.5 × 106 macrophage-specific STAT3+/+ and STAT3−/− BMDMs were unstimulated or stimulated with LPS (50 ng/ml), IFN-α (250 U/ml), or IL-27 (10 ng/ml) for 3 h. RNA was harvested, and relative IL-10 transcript was detected via qPCR normalized to L32. Data represent two independent experiments. *p < 0.05. D, ChIP using Abs against STAT1, STAT2, STAT3, c-Maf, and Sp1 using primers specific to the IL-10 promoter. Transcription factor enrichment presented as percent of input representing two independent experiments. E, Model of LPS-mediated induction of IL-10 gene expression involves sequential induction and signaling type I IFN followed induction and signaling by IL-27 signaling in macrophages.

Discussion

The innate immune system is the first line of defense against infection. However, uncontrolled production of proinflammatory of proteins is not required for IL-27-mediated IL-10 induction. We performed ChIP in BMDMs stimulated with LPS, IFN-α, and IL-27 using two sets of IL-10 promoter-specific promoters, one pair spanning the transcription start site and one pair to amplify the more distal regulatory region identified in our promoter activity analyses (−1324/-1319 bp). We show that both STAT1 and STAT3 are mobilized to the promoter in response to LPS, IFN-α, and IL-27, suggesting that they may directly regulate IL-10 promoter activity (Fig. 6D). C-Maf was recruited to the promoter by LPS, IFN-α, and IL-27 as well. Sp1 was shown to be constitutively bound at the IL-10 promoter with minimal enrichment upon stimulation, consistent with previous studies (23). In contrast, STAT2 is not recruited to the IL-10 promoter under any of the conditions tested, which is consistent with a nonessential role in IL-27-mediated IL-10 induction.
cytokines may initiate or exacerbate harmful inflammatory or autoimmune responses. IL-10 signaling represents one important source of such regulation. In macrophages, production of IL-10 is generated through TLR activation as a means of providing such a feedback response. In this study, we describe a signaling pathway that couples LPS stimulation of macrophages to IL-10 expression through the sequential generation and signaling of type I IFNs and IL-27, respectively. Our studies using LPS, IFN-α, and IL-27 CM to assess IL-10 expression suggest that the generation of signaling through these downstream gene products in response to LPS prior to IL-10 expression has physiological relevance in macrophage function.

In the past, identifying important transcription factors that mediate LPS upregulation of IL-10 has been difficult. A number of factors have been proposed to play a role in LPS-mediated IL-10 induction including IRFs, STATs, and NF-κB; however, to date, only one factor, Sp1, has proven to be essential (30, 34). One potential confounding element that may obscure the relative importance of specific transcription factors or regulatory motifs in mediating IL-10 induction by LPS is the multiple gene programs and autocrine/paracrine signaling pathways induced by LPS through TLR4. That is, although a specific transcription factor or cis regulatory element may play an important role in LPS-mediated IL-10 induction, its relative importance may be obscured through downstream events that provide compensatory or alternative means of gene activation. For example, we show that STAT1 is mobilized to the IL-10 locus in response to LPS, but we observe only partial and insignificant defects in IL-10 induction in macrophages deficient in STAT1 compared with wild-type. However, the role of STAT1 in IL-10 regulation is demonstrably clearer as we study its function in signaling pathways downstream of TLR4, like type I IFN and IL-27 (Fig. 6B). Because LPS-induced IL-10 expression indirectly occurs through the generation of secondary and tertiary signaling modules, the identification of important regulatory motifs may be obscured by these alternative or compensatory mechanisms.

In contrast, we demonstrate that IL-27–mediated IL-10 expression is direct in that it does not require the synthesis of a downstream signaling molecule or transcription factor prior to generation of IL-10 transcripts. Thus, we are more likely to identify functional cis elements within the IL-10 promoter in response to IL-27 unmitigated by additional downstream signaling events. Through promoter-reporter assays, we identify three major IL-27 response elements located at positions −1324/−1319, −510/−504, and −90/−78 relative to the transcription start site. The −90/−78 region is essential for LPS-, IFN-α-, and IL-27–mediated IL-10 promoter activity and corresponds to a previously identified Sp1 binding site (30). The −510/−504 site corresponds to a previously described c-Maf site (37). The −1324/−1319 and −510/−504 IL-27 responsive elements map larger promoter regions that correlate with LPS and IFN-α functional elements, respectively, from our promoter deletion studies, which is consistent with a downstream requirement for IL-27 signaling for LPS- and IFN-α–mediated IL-10 induction. Discrepancies between the relative roles for each element in IL-10 gene regulation may be due to the activation of alternative signaling pathways by LPS and IFN-α (34). For instance, it has been shown that maximal induction of IL-10 by LPS requires both MyD88 and TRIF adaptor molecules, whereas type I IFN production by LPS occurs solely through the TRIF signaling pathway (34).

We demonstrate that LPS, IFN-α, and IL-27 all activate and require both STAT1 and STAT3. Previous studies have suggested a role for both STAT1 and STAT3 in regulating of IL-10; however, direct binding of either factor to cis elements within the promoter have yet to be demonstrated (38). In this study, we show that both STAT1 and STAT3 as well as c-Maf are mobilized to the IL-10 promoter upon stimulation with LPS, IFN-α, and IL-27; however, we have not been able to map this binding to a specific region, though the −1319/−1324 region corresponds to a putative STAT3 site. Importantly, this site appears to be conserved from human to mouse. In addition, the site is physically distinct from a previously described IRF1/STAT3 composite site identified as a functional regulatory element in IFN-α–mediated IL-10 induction in human monocytes (38). Sp1 remains basally associated with the IL-10 promoter and is not enriched upon stimulation. It is possible that recruitment of STAT1 and STAT3 occurs only in the context of chromatin, as the IL-10 locus undergoes extensive epigenetic regulation (39–41).

In addition, it is important to note that whereas LPS, IFN-α, and IL-27 all exhibit STAT1- and STAT3-dependent activation of IL-10, the relative dependence on STAT1 and STAT3 differs among the three stimuli. Specifically, IL-27–mediated IL-10 induction exhibits an absolute requirement for STAT1 and STAT3 activity, whereas LPS and IFN-α display partial dependence on STAT3 and STAT1, respectively (Fig. 6B, 6C). Discrepancies between the relative importance of subsets of transcription factors, such as STAT1 and STAT3, as well as the relative use of cis elements within IL-10 promoter upon induction by LPS, IFN-α, and/or IL-27 highlight both the significance of the IL-27 pathway in LPS- and IFN-α–dependent IL-10 gene regulation, but also reveal a level of discordance in that both LPS and IFN-α appear to use alternative sets of signaling pathways and transcription factors to achieve maximal transcriptional induction of IL-10 (Fig. 6E) (34).

IL-27 has been implicated in both pro- and anti-inflammatory contexts. It is required for clearance of intracellular pathogens but also plays a role in inhibition of Th1/Th17 differentiation and confers a protective effect in the development of experimental allergic encephalomyelitis (15, 19, 42, 43). In this study, we show that IL-27 can contribute to a global anti-inflammatory state through the induction of IL-10 in macrophages. This is in contrast to one report in which IL-27 negatively regulates IL-10 induction in human monocytes and BMDMs (44). However, in that study, IL-27 downregulation of IL-10 occurred only when macrophages were preactivated with TLR2 or TLR4 agonists. How LPS and type I IFNs induce IL-27 expression remains to be elucidated and requires extensive promoter studies of the p28 and EBI3 loci that make up the functional IL-27 heterodimer. We show that LPS-mediated p28 induction is type I IFN dependent, consistent with studies of IL-27 regulation in human dendritic cells (45, 46). Induction of p28 expression appears to be NF-κB and IRF1 dependent (45). Further investigation into the regulation of IL-27 gene expression will undoubtedly reveal insights into the regulation of macrophage anti-inflammatory gene programs.

Acknowledgments

We thank Dr. Bin Gao (National Institute of Alcohol Abuse and Alcoholism) for providing bone marrow for macrophage-specific STAT3<sup>flox/flox</sup> LysCre<sup>+</sup> and STAT3<sup>−/−</sup> (STAT3<sup>flox/flox</sup> LysMCre<sup>−/−</sup>) and STAT3<sup>−/−</sup> (STAT3<sup>flox/flox</sup> LysMCre<sup>−/−</sup>) used in this study. We also thank Dr. Steve Smale (MIMG, University of California, Los Angeles) for helpful feedback and members of the laboratory for engaging discussion.

Disclosures

The authors have no financial conflicts of interest.

References


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