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The Protooncogene c-Maf Is an Essential Transcription Factor for IL-10 Gene Expression in Macrophages

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IL-10 is an important immunoregulatory factor. However, our understanding of IL-10 gene regulation remains very limited. In this study, following up on our previous novel finding that the protooncogene c-Maf of the basic leucine zipper family of transcription factors is expressed in monocytes and macrophages, we investigate the role of c-Maf in the transcriptional regulation of IL-10 and the underlying molecular mechanism in macrophages. c-Maf-null macrophages exhibit strongly impaired IL-10 protein production and mRNA expression upon LPS stimulation. Ectopic expression of c-Maf stimulates not only exogenously transfedct IL-10 promoter-driven luciferase activity in a dose-dependent manner but also enhances endogenous IL-10 gene expression stimulated by LPS. Both in vitro and in vivo experiments identify a c-Maf response element localized to nucleotides −196/−184 relative to the transcription initiation site in the IL-10 promoter. This site represents an atypical 12-O-tetradecanoylphosphoryl diacetylglycerol-responsive element for muscularoaponeurotic fibrosarcoma recognition and functions as an enhancer element in a heterologous and orientation-independent manner. Furthermore, c-Maf is expressed constitutively in resting monocytes/macrophages. IL-4 can up-regulate c-Maf expression, its binding to IL-10 promoter, and dose dependently enhance IL-10 production induced by LPS; moreover, IL-4 failed to enhance LPS-induced IL-10 production in c-Maf-null macrophages. Taken together, these data demonstrate that c-Maf is an indispensable yet constitutive transcription factor for IL-10 gene expression in LPS-activated macrophages, and IL-4 modulates IL-10 production in inflammatory macrophages likely via its ability to induce c-Maf expression. Thus, this study uncovers a novel and important function of c-Maf in macrophages and elucidates its transcriptional mechanism in the regulation of IL-10 gene expression. The Journal of Immunology, 2005, 174: 3484–3492.
basic region leucine zipper domain transcription factors, and the cell lineage-specific targets of c-Maf have been identified; some examples include the following: IL-4 in Th2 cells (15), the crystalline genes in lens fiber cells (16, 17), insulin gene in islet β cells (18), p53 (19), and L7 (20). In all these genes, c-Maf exerts its transcriptional role through binding to a Maf recognition element (MARE) (21). There are two forms of human c-Maf mRNA, c-Maf-long and c-Maf-short, as the result of differential splicing (22, 23). The first direct in vivo demonstration of a physiological role of c-Maf was provided by studies in c-Maf-null mice (16, 17, 24). Disruption of the c-Maf gene affected both intrauterine and postnatal survival (17). In addition, it has been shown that c-Maf is one of the master regulators for Th2 differentiation (15, 25). Therefore, c-Maf is both a developmentally and immunologically important gene. In the present study, we further investigated the molecular mechanisms by which c-Maf regulates IL-10 gene transcription in macrophages.

Materials and Methods

Mice
c-Maf+/− mice were kindly provided by Dr. I. Cheng Ho of Harvard School of Public Health (Boston, MA) (17). Fetal liver-derived macrophages were prepared from gestation day 14.5 embryos by intercrossing c-Maf−/− mice as described previously (15).

Cells and reagents
RAW264.7 murine macrophage cell line (ATCC TIB-71), human monocytic cells, and culturing were conducted as described previously (26). LPS (Escherichia coli serotype 0127:B8) was purchased from Sigma-ALdrich (catalog no. L-3129). The Abs were as follows: c-Maf (catalog no. A300-613A; Bethyl Laboratories), c-Maf (M153, sc-7866x; Santa Cruz Biotechnology), and c-fos (sc-52x; Santa Cruz Biotechnology). Recombinant human IL-4 and IFN-γ were purchased from Genzyme.

Reporter plasmids
The human IL-10 promoter-luciferase construct (pIL-10 (−1044/+30)-luc) was generously provided by Dr. L. Zaegler-Henrikson of University of Leicester (Leicester, U.K.) (27). For generation of the 5′-successive deletion series, the upstream and downstream PCR primers were designed to include BamHI and XhoI restriction sites, respectively. The PCR products were ligated directly into pcR2.1 T/A vector and then subcloned into the same backbone of pIL-10 (−1044/+30)-luc plasmid by using BamHI/XhoI digestion. For the substitution mutants, an overlapping PCR procedure was used (28) by using a pair of overlapping internal primers that contain the mutagenic sequences. All constructs are relative to the transcriptional initiation site and confirmed by sequencing against the human IL-10 promoter (GenBank accession no. Z30175; Ref. 29). The murine IL-4 promoter-driven luciferase construct, pIL-4 (−157/+68)-luc, was kindly provided by Dr. Richard Flavell of Yale University (New Haven, CT) (15). For the chimeric promoter constructs, the consensus MARE, 5′-TCCTTTTTGCTTACGATGCAAAAATTGAAAACTA-3′, and antisense (−147/−132), 5′-CTTTTAGGGCTCCTCTCT-3′ (65-bp product). As a negative control, a separate region of the human IL-10 promoter was also included in the ChIP experiment. It is located between −3158 and −2947 upstream of the −206/−132 region. The pair of PCR primers used in this control were sense, 5′-AGTGAAGAGCCGGCCACCTA-3′, and antisense, 5′-ATCCCCACTGGAAATTTCC-3′. The PCR cycles were as follows: 95°C for 3 min, 60°C for 40 s, and 72°C for 30 s, 1 cycle; 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, 29 cycles; and 72°C for 7 min, 1 cycle.

RNase protection assay (RPA)
Total RNA was purified using Ultraspec RNA isolation system (Biotec Laboratories). RPA was performed by using the Riboquant kit (BD Pharmingen), according to the manufacturer’s instructions. The mCK-2b and hCK2 template sets were used. The respective cytokine mRNAs were quantified by using the PhosphorImager and ImageQuant (ImageQuant 5.0; Molecular Dynamics) and normalized to the corresponding L32 and GAPDH controls.

Cytokine production by ELISA
BD Pharmingen OptEIA sets for mouse IL10, human IL-10, and human IL-12p40 were used.

Nuclear extract and recombinant human c-Maf
Nuclear extracts were isolated as described previously (33). The recombinant human c-Maf protein was synthesized by using pT3/T Quick Coupled transcription/Translation System (Promega). In brief, both human c-Maf-long and -short form cDNA were cloned individually into pT3/T vector under the T7 promoter and named pT3/Tc-Maf and then proceeded to the pT3/T reaction following the instruction.

EMSA
EMSA, competitive EMSA, and supershifts were performed as described previously (34). The following oligonucleotides were used as probes: MARE consensus, 5′-GGATGGCATCAGCATTCAACTTAC-3′ and 5′-TCATTGGTCTGGTGTTTATCAGGACGAC-3′; and 5′-203/−170 wild type, 5′-GGTGAGGGGACGGCGCTTTCA-3′, and 5′-203/−170mutant (M7), 5′-TAATTTTTGGAGGTCTGGTGTTTATCAGGACGAC-3′ (core element underlined); and NF-κB consensus, 5′-AGTGGAGGGGACGGCGCTCTCA-3′.

Computational analysis of the IL-10 promoter region
Human IL-10 promoter region −1044/+30 was analyzed by the MatInspector V2.2 professional program (35) to predict transcription factor binding sites.

RT-PCR analysis of gene expression
Reverse transcription reactions were performed as previously described (13). The following primers were used for PCR: human IL-10 (GenBank accession no. M57627) forward (position 104–129), 5′-AGCTTGGTCTGGTGTTTATCAGGACGAC-3′ and reverse (position 297–318), 5′-GGTGGCATCAGCATTCAACTTAC-3′ (215-bp product, annealing temperature 60°C, 30 cycles). Because the cDNA sequences of c-Maf-long and c-Maf-short are identical until the position 1925 (22), the same upstream PCR primer for the two forms of c-Maf was used: sense (position 1571–1592), 5′-TAATTTTTGGAGGTCTGGTGTTTATCAGGACGAC-3′; antisense: short form (position 1957–1938), 5′-GGTGAGGGGACGGCGCTCTCA-3′ (core element underlined); and NF-κB consensus, 5′-AGTGGAGGGGACGGCGCTCTCA-3′.
activity (Fig. 2A) introduced c-Maf dose dependently up-regulated the IL-10 promoter cytokine gene expression (36, 37). The result shows that ectopically human c-Maf expression plasmid into the mouse macrophage cell line RAW264.7, which has been used extensively to study macrophage (53x304) human c-Maf expression plasmid into the mouse macrophage cell line (53x404) production alone did not induce the endogenous IL-10 gene transcription. One representative of five embryos for each group (53x135) was used. Human monocyte-derived macrophages were transduced with adenovirus-expressing human c-Maf or EGFP (as control). Twenty-four h after the transduction, the macrophages were treated with LPS for 4 h or primed with human IFN-γ for 16 h, then treated with LPS for 4 h. The endogenous IL-10 mRNA expression was detected by RT-PCR (Fig. 1B) and RPA (Fig. 1C). Compared with the control adenovirus, c-Maf transduction of primary macrophages significantly enhanced LPS- or IFN-γ/LPS-stimulated IL-10 mRNA expression (compare lanes 2 and 3 to 4 and 5, respectively, in Fig. 1, B and C). c-Maf adenovirus transduction alone did not induce the endogenous IL-10 gene transcription (Fig. 1A, lane 1).

Ectopic c-Maf expression can activate IL-10 gene transcription

Additionally, to demonstrate the transcriptional role of c-Maf in the regulation of IL-10 gene expression, the human IL-10 promoter (−1044/+30)-driven luciferase reporter was cotransfected with the human c-Maf expression plasmid into the mouse macrophage cell line RAW264.7, which has been used extensively to study macrophage cytokine gene expression (36, 37). The result shows that ectopically introduced c-Maf dose dependently up-regulated the IL-10 promoter activity (Fig. 2A). The short and long forms of c-Maf worked at similar efficiencies in activating the IL-10 promoter activity (Fig. 2B). Thus, the c-Maf-long form was used for all subsequent experiments.

Next, we wanted to define the role of the endogenous c-Maf in IL-10 transcription. To address this issue, we used the well-characterized v-Maf-DNM (38). Protein blast analysis (www.ncbi.nlm.nih.gov/BLAST/) indicated that the bZIP-Maf identity between v-Maf (GenBank accession no. NP062191) and mouse c-Maf (GenBank accession no. I57555) or human c-Maf-short form (GenBank accession no. AAC27037) or human c-Maf-long form (GenBank accession no. AAC27038) is 98, 99, and 98%, respectively. These high degrees of homology suggest that v-Maf-DNM may be able to functionally block mammalian c-Maf. To verify whether the v-Maf-DNM, Mxi-v-Maf, could block the function of mammalian c-Maf, we first tested its activity on a mouse IL-4 promoter-driven luciferase reporter that has been shown to respond to c-Maf (15). The cotransfection of pIL-4 (−157/+68)-lac with c-Maf or c-Maf plus Mxi-v-Maf into RAW264.7 cells showed that Mxi-v-Maf strongly blocked the c-Maf-induced IL-4 transcription (data not shown), confirming its cross-species efficacy. We then cotransfected the pIL-10(−1044/+30)-lac with c-Maf or c-Maf plus Mxi-v-Maf. As shown in Fig. 2C, Mxi-v-Maf completely blocked ectopic c-Maf-induced IL-10 transcription. Moreover, when we cotransfected pIL-10(−1044/+30)-lac with Mxi-v-Maf into RAW264.7 cells, it also completely inhibited the constitutive as well as LPS-induced IL-10 promoter activity (Fig. 2D), suggesting that the endogenous c-Maf present in RAW264.7 cells (13) is not only required for LPS-induced IL-10 gene expression but also contributes to the basal level of IL-10 transcription.

Localization of the c-Maf response region in human IL-10 promoter

To identify the c-Maf response element(s) in the IL-10 promoter, a series of the 5′ deletion mutants of IL-10 promoter-driven luciferase constructs were cotransfected with the c-Maf expression plasmid into RAW264.7 cells, and the luciferase activities were assayed relative to the activity of the full-length promoter (−1044/+30), which was set arbitrarily as 100%. Two constructs, −408 (no. 8) and −171 (no. 12) (Fig. 3), showed considerable decrease in their response to c-Maf, suggesting that the regions −511/−408 and −206/−171 may harbor c-Maf-responsive element(s). The complete loss of promoter activity in the −61 construct (no. 16) was likely due to the fact that the truncation was in the proximity of TATA-box, which is located at −59/−54 (see Fig. 5A for details).
c-Maf binds to the −206/−171 region in the IL-10 promoter in vitro

The above 5’ deletion mutants have narrowed down the c-Maf-responsive elements to two possible regions on the IL-10 promoter. We then localized the exact c-Maf binding site on the IL-10 responsive elements to two possible regions on the IL-10 promoter. The full-length (−1044/+30) and a series of 5’ deletion mutants of the human IL-10 promoter-luciferase constructs were cotransfected with c-Maf expression vector into RAW264.7 cells at a molar ratio of 1:14 (reporter:effector). The relative luciferase activities were plotted against that of the −1044/+30 promoter, which is set as 100%. A representative of two experiments with similar results are shown.

FIGURE 2. Ectopic c-Maf expression activates IL-10 gene transcription. A. RAW264.7 cells were transfected by electroporation using 6.5 μg of pIL-10 (−1044/+30)-luc reporter construct together with indicated amounts of expression vector c-Maf (long form) or its control vector pCEFL. The total amount of the effector plasmids (pCEFL/c-Maf) was maintained at 1.8 μg with pCEFL. The result shown here is the summary of two independent experiments (mean ± SD). B, The long and short forms of c-Maf were cotransfected with the IL-10 promoter as described in A. Their respective luciferase activities were expressed as fold induction compared with the activity of pCEFL plus IL-10 promoter, which was set as 1. C, RAW264.7 cells were cotransfected with 6.5 μg of pIL-10 (−1044/+30)-luc with control vectors or pIL-10 (−1044/+30)-luc plus 1.8 μg of c-Maf expression vector (reporter:effector = 1:1/4 in molarity) or pIL-10 (−1044/+30)-luc and c-Maf expression vector plus v-Maf-DNM (reporter:effector: Mxi-v-Maf = 1:1/4:1 in molarity). Luciferase activity was expressed as fold induction (relative luciferase activity) by arbitrarily setting the activity of pIL-10 (−1044/+30)-luc as 1. Statistical analysis indicated that c-Maf significantly up-regulated IL-10 promoter-driven luciferase activity (p < 0.001, lane 1 vs 2), and Mxi-v-Maf completely blocked the exogenously transfected c-Maf on the induction of pIL-10 (−1044/+30)-luc (p < 0.001, lane 2 vs 3; p > 0.05, lane 1 vs 3). D, RAW264.7 cells were cotransfected with 6.5 μg of pIL-10 (−1044/+30)-luc with control vector or with Mxi-v-Maf. Transfected cells were either left alone (medium) or treated with 1 μM/ml LPS for 20 hr. Statistical analysis indicated that Mxi-v-Maf significantly blocked the endogenous c-Maf-induced basal luciferase activity of pIL-10 (−1044/+30)-luc (p < 0.05, lane 3 vs 4). One representative of three independent experiments is shown.

FIGURE 3. Identification of c-Maf-responsive regions in the IL-10 promoter. The full-length (−1044/+30) and a series of 5’ deletion mutants of the human IL-10 promoter-luciferase constructs were cotransfected with c-Maf expression vector into RAW264.7 cells at a molar ratio of 1:14 (reporter:effector). The relative luciferase activities were plotted against that of the −1044/+30 promoter, which is set as 100%. A representative of two experiments with similar results are shown.
FIGURE 4. Binding of c-Maf to IL-10 promoter in vitro. A and B, Recombinant human c-Maf-long protein, generated by the PTNT system, was used in EMSA with $^32$P-labeled $\sim203/-170$ sequence from the IL-10 promoter (A) or the MARE consensus (B). Competition for c-Maf binding was done by adding cold $\sim203/-170$ or MARE or the NF-kB consensus sequences in 50× molar excess. The terms “self” and “non-self” refer reciprocally to $\sim203/-170$ and MARE, respectively. Lane 6 in A and B is mock transcription-translation controls. C, A series of consecutive mutant probes of every 4- to 5-bp mutations (M1–M6) or a 13-bp mutation (M7) of the predicted c-MARE were generated in the context of the $\sim203/-170$ sequence (mutated sequences are in boldface and underlined). D, The mutant oligos described in C were used as cold competitors (50× molar excess) in competitive EMSA with the wild-type $\sim203/-170$ probe and recombinant human c-Maf protein. E, Supershift EMSA was conducted with the $\sim203/-170$ probe and recombinant human c-Maf protein. Anti-c-Maf Abs (Bethyl Laboratories and Santa Cruz Biotechnology; Fig. 4, lanes 2 and 3, respectively) but not of anti-c-fos Ab were used. F and G, Supershift EMSA was conducted using the $\sim203/-170$ probe and the nuclear extract isolated from RAW264.7 cells, which had been transfected with the c-Maf expression vector for 48 h, followed by 4 h of LPS stimulation (1 μg/ml; F) or nuclear extract isolated from HEK 293 cells transduced with c-Maf-expressing adenovirus (G). The same Abs were used as in E. The supershifted bands are indicated by an asterisk (lane 3 in E; lane 2 in F).

Specific in vivo c-Maf binding to the IL-10 promoter in human macrophages

To determine whether c-Maf could bind to the MARE-like sequence in the IL-10 promoter in vivo, ChIP assay was performed. This technique can establish whether a known transcription factor binds in the vicinity of a known regulatory element in living cells (39). As Fig. 5 demonstrates, in primary human monocyte-derived macrophages, c-Maf specifically bound to the IL-10 promoter in this region in vivo between $\sim206$ and $\sim132$ (Fig. 5B) but not to a separate region 3 kb upstream (Fig. 5C). Surprisingly, the binding was not inducible by LPS stimulation over a period of 30 h (Fig. 5D). The above data collectively suggest that c-Maf can bind to the $\sim203/-170$ sequence both in vitro and in vivo, and the core element for this binding resides in the 13-bp region at $\sim196$ to $\sim184$ in human IL-10 promoter.

The $\sim196/-184$ sequence acts as an enhancer

The above results provided evidence that $\sim196/-184$ in human IL-10 promoter is a MARE-like sequence. To test the function of this element in the induction of IL-10 promoter activity by c-Maf, a reporter construct with a 13-bp substitution (M7) was constructed on the backbone of $\sim206/+/30$ of the IL-10 promoter. Compared with pIL-10 (−206)-luc, the response of pIL-10 (−100)(−206)M7-luc to c-Maf was decreased by $>50$% and down to the level of the $\sim171/+/30$ construct (Fig. 6A). Additionally, to characterize the function of this c-Maf response element, chimeric promoters were generated (Fig. 6B). Transient transfection of these constructs with c-Maf expression plasmid was performed in RAW264.7 cells. As shown in Fig. 6B, in the forward orientation, although the wild-type $\sim206/-170$ sequence, which contains the MARE-like element, was less potent in the response to c-Maf than that of the consensus MARE-containing TK promoter, the 13-bp M7 mutation resulted in a complete loss of its c-Maf response, decreasing to the basal level (Fig. 6B, upper panel). In the reverse orientation, although wild-type $\sim206/-170$ TK chimeric promoter showed an even greater response to c-Maf, the M7 mutant was still nonresponsive (Fig. 6B, lower panel). Taken together,
Furthermore, IL-4 added at p70 (data not shown) synthesis, which is consistent with our pre-
lLPS strongly enhanced IL-12 p40 and (Fig. 7A to LPS. There was a differential impact of the IL-4 exposure on 
ulate c-Maf expression in human monocytes/macrophages? 2) Does IL-4 mod-
tissue injury repair (40, 41). On the basis of the established role of 
flammatory macrophages can be deactivated by IL-4 or IL-13 
scription regulation, i.e., under what circumstance(s) is this c-Maf-
these data additionally demonstrate that the 13-bp MARE-like se-
sequence is a functional c-Maf response element; moreover, because it 
works in an orientation-independent manner, it may act as an 
enhancer element for IL-10 transcription.

**IL-4 enhances c-Maf expression and IL-10 production**

Additionally, we explored the physiological relevance of this 
scription regulation, i.e., under what circumstance(s) is this c-Maf-
ated transcriptional regulation invoked? It is well known that 
flammatory macrophages can be deactivated by IL-4 or IL-13 and 
play important roles in humoral immunity, homeostasis, and 
tissue injury repair (40, 41). On the basis of the established role of 
IL-4 in transcriptional regulation of c-Maf in Th cells (42, 43), we 
asked the two following questions: 1) Does IL-4 influence IL-10 
production in human monocytes/macrophages? 2) Does IL-4 mod-
ulate c-Maf expression in human monocytes/macrophages?

To address the first question, human monocytes were treated 
with LPS or LPS + IL-4 and added at different time points relative to LPS. There was a differential impact of the IL-4 exposure on 
LPS-induced IL-10 and IL-12 p40 production. Addition of IL-4 
together or 2 h after LPS stimulation enhanced IL-10 production (Fig. 7A) while strongly inhibiting IL-12 p40 production (Fig. 7B). 
IL-4 added at 16 h before LPS strongly enhanced IL-12 p40 and 
p70 (data not shown) synthesis, which is consistent with our 
previous report of the priming effect of IL-4 on IL-12 production (44). 
Furthermore, IL-4 added at +2 h dose dependently enhanced 
LPS-induced IL-10 production (Fig. 7C) while strongly suppressing 
IL-12 p40 production (Fig. 7D). These results are consistent with 
the reported effects of IL-4 on endotoxin-induced IL-10 production in murine macrophages (45). To address the second question, we 
examined the effect of IL-4 on the regulation of c-Maf mRNA 
expression. Human monocytes were treated with IL-4 in different 
doses for 2 h, and the total RNA was isolated for RT-PCR. Fig. 7E 
shows that IL-4 dose dependently stimulated c-Maf expression 
(both short and long forms) in resting monocytes, indicating that, 
as with Th cells (42, 43), IL-4 regulates c-Maf transcription also in 
macrophages. Control experiments in the nonadherent portion of the human PBMC with the same treatment indicated that the c-Maf 
mRNA expression observed was derived predominantly from 
monocytes and not from lymphocytes (data not shown). Addition-
ally, we characterized the effect of LPS, IL-4, and LPS + IL-4 on 
the c-Maf mRNA expression. Human monocytes were treated with 
LPS, IL-4, or LPS + IL-4 for 8 h (at this time point, IL-10 mRNA 
reaches its peak (data not shown)). The total RNA was isolated for 
RT-PCR. Fig. 7F shows the following: 1) c-Maf was expressed 
constitutively in resting monocytes; 2) at 8 h, LPS did not stimu-
late an enhancement in c-Maf transcription; and 3) IL-4 
treatment enhanced and IL-4 + LPS further augmented c-Maf 
expression. ChIP assay in human monocytes also showed that IL-4 
could by itself induce endogenous c-Maf binding to the IL-10 pro-
moter at the MARE, even to a greater extent than LPS (compare 
lane 3 to lane 2 of Fig. 7G), and the LPS + IL-4 combination had an 
additive effect (Fig. 7G, lane 4). An important point to note is 
that although IL-4 alone stimulates high c-Maf expression, it is 
sufficient to induce IL-10 production by itself, and LPS treatment 
is required (Fig. 7A). This observation is consistent with the data 
shown in Fig. 1B, where we found that c-Maf adenovirus-mediated 
c-Maf overexpression alone did not induce IL-10 gene expression. 
Only by working together with LPS can the endogenous IL-10 
gene be turned on. Furthermore, to investigate the role of c-Maf in 
IL-4-enhanced IL-10 expression, c-Maf-null macrophages were
c-Maf

natants were collected for IL-10 (A and B) or IL-12 p40 (relative to LPS. Twelve hours later, cell culture supernatants were collected for IL-10 (A and B) or IL-12 p40 secretion by ELISA. * A value of $p < 0.05$ compared with LPS treatment alone. C and D, Human monocytes were treated with different doses of rhIL-4 and added 2 h after LPS stimulation. Cell culture supernatants were collected 12 h after LPS stimulation for IL-10 and IL-12 p40 secretion by ELISA. * A value of $p < 0.05$ and * $p < 0.01$, compared with LPS treatment alone. E, Human monocytes were treated with different concentrations of IL-4 as indicated for 2 h, and total RNA was isolated for RT-PCR analysis of c-Maf expression (both short and long forms). GAPDH was analyzed as the internal control. F, Human monocytes were treated with IL-4 (1 ng/ml) or LPS (1 $\mu$g/ml) or both (IL-4 was added together with LPS) for 8 h, and total RNA was isolated for RT-PCR to examine the long- and short-form c-Maf mRNA expression. GAPDH was analyzed as the internal control. G, Human monocytes were treated with recombinant murine IL-4 (1 ng/ml) or LPS (1 $\mu$g/ml) or both (IL-4 was added at $+2 h$) for 8 h, and ChIP assay was conducted as described in Fig. 5B. H, Fetal liver-derived macrophages from c-Maf$^{+/−}$ and c-Maf$^{−/−}$ littermate embryos of gestation day 14.5 were plated into 48-well plates at a density of $0.2 \times 10^{6}$ cells/ml/well. Cells were stimulated with 1 $\mu$g/ml LPS, murine IL-4 (1 ng/ml), or LPS + IL-4 (added at $+2 h$). Supernatants were collected 24 h after LPS treatment for ELISA. Results shown are the summary of four heterozygous and three homozygous knockout embryos. ND, nondetectable.

treated with LPS, IL-4, or LPS + IL-4. As shown in Fig. 7H, LPS-induced IL-10 production was enhanced strongly by IL-4 in cells heterozygous for c-Maf ($p = 0.0086$) but not in c-Maf$^{−/−}$ littermate-derived cells ($p = 0.1453$), demonstrating that c-Maf is at least partially required for IL-4-enhancement of LPS-induced IL-10 production.

**Discussion**

Upon LPS stimulation, the kinetics of cytokine production in macrophages is characterized by rapid induction of proinflammatory cytokines and followed by the production of anti-inflammatory cytokines, e.g., IL-10, hence, dampening the exaggeration of proinflammatory cytokine production and associated pathology. This kinetic cytokine production pattern is illustrated in the sepsis syndrome in vivo (46). IL-10, as a potent anti-inflammatory cytokine, inhibits the expression of a number of inducible genes in macrophages, including several proinflammatory cytokines (47). Despite its biological importance, relatively little is known about the IL-10 gene regulation. In the present study, we report that protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. There are four sets of experiments that point to this conclusion. First, c-Maf-null macrophages exhibit impaired IL-10 production upon LPS stimulation. Second, c-Maf up-regulates both the exogenous IL-10 promoter activity, as well as the endogenous IL-10 gene transcription stimulated by LPS. Third, a c-Maf binding site is localized in the IL-10 promoter to $−196/−184$, which resembles a TRE-type MARE, demonstrated by both in vitro (EMSA) and in vivo (ChIP assay) experiments. Fourth, functional studies reveal that this site is operational in the response to c-Maf induction by mutagenesis and chimeric promoter approaches. All of these results indicate that c-Maf is a nonredundant transcription factor and regulates IL-10 gene expression through physically binding to the IL-10 promoter. Transcriptional regulation is dependent on the interaction among transcription factors and cis-elements. In addition, the cis-elements are wrapped by histones and not always accessible by the transcription factors or transcription factor complexes. Therefore, modification of histones determines their state—loose or condensed—and then determines the accessibility of these cis-elements and then determines the gene transcription state (histone codon theory). The observation that c-Maf is expressed constitutively in resting macrophages initially prompted us to postulate that c-Maf may not bind the MARE-like site in IL-10 promoter until later time points after LPS stimulation, which is known to induce chromatin changes (48). The ChIP analyses surprisingly revealed constitutive c-Maf binding to the MARE sequence in both the resting state and LPS-activated human macrophages (Fig. 5B, B and C). There are a few possible explanations: firstly, c-Maf is constantly present and active, and LPS stimulation induces additional factors that, together with c-Maf, contribute to transcriptional activation of IL-10 in
macrophages. Secondly, c-Maf may undergo posttranslational modifications. It is reported that c-Maf activity is regulated negatively by the protein kinase A-, protein kinase C-, and MAPK/ERK-signaling cascades; however, mutation of the two most likely phosphorylation sites didn’t change its transcription activity (49). Thirdly, c-Maf can interact with transcriptional coactivators CREB-binding protein/p300, as shown in the case of a-crystallin promoter. The coactivation of c-Maf by CREB-binding protein/p300 requires histone acetyltransferase activity for crystalline gene expression (50), and their recruitment could modulate the chromatin structure, exposing the cis-elements for access by LPS-induced transcription factors and then turn on the IL-10 gene.

Gene regulation is a complex process that involves the coordinated integration of distinct signal transduction pathways. Although c-Maf is essential for IL-10 transcription, c-Maf itself is not enough, and its ability to do so still requires LPS stimulation. This situation is analogous to many other factors that have been shown to be involved in the transcription of IL-10, e.g., CREB-1, activating transcription factor-1 (51, 52), C/EBP (53), SV40 promoter 1 (6, 54), SV40 promoter 55 (55), and STAT3 (27). All these factors require an accompanying LPS stimulation, suggesting that multiple signal transduction pathways are involved in the activation of IL-10 expression (56). Furthermore, LPS-induced IL-10 gene transcription requires de novo protein synthesis because cycloheximide treatment before LPS stimulation can completely block IL-10 gene induction (6). Thus, we think that c-Maf works in concert with LPS-induced, yet-to-be-identified factors to activate the IL-10 gene. In support of this hypothesis, blocking AP-1 or CREB by gene induction (6). Thus, we think that c-Maf works in concert with LPS-induced, yet-to-be-identified factors to activate the IL-10 gene. In support of this hypothesis, blocking AP-1 or CREB by gene induction (6). Thus, we think that c-Maf works in concert with LPS-induced, yet-to-be-identified factors to activate the IL-10 gene. In support of this hypothesis, blocking AP-1 or CREB by gene induction (6). Thus, we think that c-Maf works in concert with LPS-induced, yet-to-be-identified factors to activate the IL-10 gene.

There is increasing evidence indicating that macrophages are a diverse and dynamic population of cells that can be activated through a number of distinct pathways and participate in a wide range of critical functions (41). In this study, we demonstrated that the IL-10-enhancing effect of IL-4 in LPS-activated human monocytes (Fig. 7, A and C) is similar to that shown in mouse macrophages (45). Then we investigated the modulation of c-Maf by IL-4 in human monocytes because it was reported that IL-4 could up-regulate c-Maf through Stat6 in Th cells (42). Our data demonstrate that IL-4 can regulate c-Maf expression and its binding to IL-10 promoter in human macrophages. Moreover, the characteristics of IL-4-enhanced IL-10 production (Fig. 7C) not only correlate well with its ability to stimulate c-Maf expression (Fig. 7E) in human monocytes but also show direct dependency on c-Maf (Fig. 7H). Considering that c-Maf is an essential transcription activator for IL-10 gene transcription, our data suggests that IL-4, which is derived mainly from activated Th2 cells, may exert its macrophage-regulating activity through stimulation of c-Maf. This idea is supported by the c-Maf overexpression experiment in human monocyte-derived macrophages, in which c-Maf overexpression enhanced LPS-induced IL-10 expression (Fig. 1, B and C), and by the study in c-Maf-null macrophages where Rl–/– failed to enhance LPS-induced IL-10 production (Fig. 7H).

Inflammation is a complex response, and it is an attempt to restore homeostasis (58). Macrophages are one of the main players in inflammatory immune responses. In this study, we demonstrated that c-Maf is an essential transcription factor for IL-10 production in inflammatory macrophages. This observation, together with our previous report that c-Maf is an inhibitor of IL-12 production, suggests that it may be an important factor in the homeostatic regulation. Our findings are one step further to understanding the intriguing and complex mechanisms of macrophage-gene regulation.
REGULATION OF IL-10 EXPRESSION BY c-Maf IN MACROPHAGES


