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J Immunol 2002; 169:5715-5725; doi: 10.4049/jimmunol.169.10.5715
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Differential Regulation of IL-12 and IL-10 Gene Expression in Macrophages by the Basic Leucine Zipper Transcription Factor c-Maf Fibrosarcoma

Shanjin Cao,* Jianguo Liu,* Marta Chesi,† Peter Leif Bergsagel,† I-Cheng Ho,‡ Raymond P. Donnelly,§ and Xiaojing Ma²*

IL-12 is a principal activator of both innate and adaptive immunity against infectious agents and malignancies. Regulation of proinflammatory IL-12 gene expression in phagocytes by the anti-inflammatory cytokine IL-10 represents a major homeostatic process underlying host-pathogen and host-self interactions. Delineation of the signaling pathway of IL-10 is crucial to the understanding of immunological regulatory networks. In this study, we report that IL-10 and c-musculoaponeurotic fibrosarcoma (Maf) induce their mutual expression in inflammatory macrophages. We demonstrate that c-Maf is one of the physiological mediators of IL-10's immunosuppressive activities. When overexpressed, c-Maf selectively inhibits transcriptional activation of IL-12 p40 and p35 genes while potently activating IL-10 and IL-4 expression, potentially contributing to the development of a state of anti-inflammation and dichotomy of immunologic polarization. c-Maf induces changes in nuclear DNA-binding activities at multiple sites including the ets, GA-12, NF-κB, C/EBP, and AP-1 elements. Nonetheless, the essential c-Maf-responsive element appears to be located elsewhere. Inhibition of IL-12 p40 gene expression by c-Maf requires the N-terminal transactivation domain, suggesting an indirect mechanism of transcriptional inhibition involving the induction of an unidentified repressor. In c-Maf-deficient murine macrophages, IL-10 production is impaired. However, IL-10-mediated inhibition of IL-12 production remains intact, indicating the existence of alternative mediators in the absence of c-Maf, consistent with the observation that a functional AP-1 is required for this pathway. The Journal of Immunology, 2002, 169: 5715–5725.

Inflammatory responses help the immune system respond to infection, while anti-inflammatory responses, arising as a byproduct, control excessive damage to the host. Loss of this balance can lead to disproportionate pathology or immunosuppression. Cytokines play major roles in regulating the type, duration, and extent of immune responses in a temporal and spatial manner. IL-10 and IL-12 are two key players in these processes, usually acting in opposition. IL-12 is a heterodimer produced primarily by macrophages and dendritic cells in both innate and adaptive immune responses and helps induce T cell-dependent and -independent activation of macrophages and NK cells, generation of Th type 1 and cytotoxic T cells, and resistance to intracellular infections (1). IL-12 has powerful antitumor and antitumorigenic activities against many murine and human tumors (2). The genes encoding the two heterologous chains of IL-12, p40, and p35 are located on different human chromosomes. The highly coordinated expression of p40 and p35 genes is essential for the initiation of an effective immune response. Infectious agents and tumors evade immune activation by producing immunosuppressive agents such as IL-10, IL-4, TGF-β, PGE2, glucocorticoids, etc. that inhibit IL-12 production. How these immune suppressants inhibit IL-12 production at the molecular level is not well understood.

We have previously demonstrated that IL-10-mediated inhibition of IL-12 production in human monocytes occurs primarily at the level of transcription of the p40 and p35 genes and requires de novo protein synthesis (3). Efforts to delineate the molecular pathway of this inhibition required identification of mediators of IL-10’s potent anti-inflammatory and immunosuppressive activities. We used cDNA microarray analysis to search for genes that were induced by IL-10 in LPS-activated human macrophages as candidates to participate in inhibition of IL-12 production. One of the genes we identified in this search was c-musculoaponeurotic fibrosarcoma (Maf).3 c-Maf is the cellular counterpart of v-Maf, the transforming gene of the avian retrovirus AS42 for Maf. c-Maf belongs to a growing family of basic leucine zipper (bZIP) transcription factors (4). Along with cyclin D1 and fibroblast growth factor receptor 3, c-Maf was identified as one of three most frequently dysregulated proto-oncogenes by chromosomal translocation to an IgH locus in human multiple myeloma (5, 6). The role of cyclin D1, fibroblast growth factor receptor 3, and c-Maf in the etiology of this malignancy has not been defined. The first direct demonstration of a physiological role of c-Maf was provided by studies in mice genetically rendered deficient in this gene (7–9). Disruption of the

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1 This work was supported by National Institutes of Health Grants AI-45899 and CA-79772 (to X.M.). S.C. is supported by a Susan G. Komen Breast Cancer Foundation Postdoctoral Fellowship.

2 Address correspondence and reprint requests to Dr. Xiaojing Ma, Department of Microbiology and Immunology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. E-mail address: xim2002@med.cornell.edu

3 Abbreviations used in this paper: Maf, musculoaponeurotic fibrosarcoma; bZIP, basic leucine zipper; RPA, RNase protection assay; RT, reverse transcription; LZ, leucine zipper domain; HO, heme oxygenase; IRES, internal ribosomal entry site; EGFP, enhanced green fluorescent protein.

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REGULATION OF IL-12 AND IL-10 GENE EXPRESSION BY c-Maf

c-Maf gene affected both intrauterine and postnatal survival (7). Subsequently, it was shown that deficiency in c-Maf resulted in a specific defect of IL-4 production by CD4+ T lymphocytes and a lack of Th2 differentiation (10). Thus, c-Maf is both a developmentally and immunologically important gene.

In this study, we demonstrate that c-Maf is a potent activator of IL-10 gene expression in monocytes/macrophages. When overexpressed, it could also suppress IL-12 p40 and p35 gene transcription. We explore the underlying molecular mechanisms.

Materials and Methods

Cells and reagents

Human monocytes were obtained by leukopheresis and the purity of the preparations was routinely >95%. The murine monocyte-like cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin. Anti-IL-10 and isotype control Abs were purchased from R&D Systems (Santa Cruz, CA). Recombinant human and murine IFN-γ were purchased from Genzyme (Boston, MA). Recombinant human M-CSF was purchased from PeproTech (Rocky Hill, NJ). Recombinant rat IL-4 was purchased from Sigma-Aldrich (St. Louis, MO). All Abs used in EMSA analysis were from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids

All human IL-12 p40 promoter-luciferase constructs have been described previously (11, 12). The human IL-12 p35 promoter was as described (13). The murine IL-4 promoter-luciferase construct was obtained from Dr. R. Flavell of Yale University (New Haven, CT; Ref. 14). The human IL-10 promoter-luciferase construct was obtained from Dr. L. Ziegler-Heitbrock of University of Leicester (Leicester, U.K.). It contains a piece of the human IL-10 promoter region up to −1044. The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA; catalog no. 219078). The human c-Maf cDNA (both long and short isoforms) was tagged with hemagglutinin and cloned into the mammalian expression vector pCEFL, under the EF-1α promoter (15). The mutant c-Maf construct bZIP domain (LZ) was generated by PCR (sense primer: GGGAATTCCTGCACTTGCAC GACGGGCTTC; antisense primer: CCCCCTAGATCACATTTGAAAACT CGGGAGAGGA). It contains the basic DNA-binding domain and the LZ, lacking the transactivation domain completely. Likewise, the LZ mutant containing only the LZ generated with the sense primer (ACGAATTCCA GGGGAATTCCTGCACTTGCAC GACGGGCTTC) and the C/EBP mutant lacking the transactivation domain (EF-1α bZIP domain) was kindly provided by Dr. C. Vinson (National Cancer Institute, NIH). C/EBP and AP-1 mutants of the human IL-10 promoter-luciferase construct were created by overlapping PCR in the region −292/−108 construct. The wild-type sequence in this region is GTTT CAATGTGTCACAAGATCGTTAG, the C/EBP mutant sequence is GTT TCTCAAATGCGATCCAAATCGTTAG, with the underlined sequence being the target site different between the two. The AP-1 mutant construct has the sequence of CACCAAGTGGTTGTACGTTAG vs the wild-type sequence CATC AAGTGGTTGTACGTTAG. All mutant constructs generated by PCR were confirmed by DNA sequencing. The dominant-negative mutant of AP-1 (κ-Bos) was generously provided by Dr. C. Vinson (National Cancer Institute, National Institutes of Health, Bethesda, MD). All plasmids were purified using the Qiagen Endotoxin free kit (Qiagen, Valencia, CA).

Adenoviral vectors and their propagation

We integrated p-internal ribosomal entry site 2 (pIRES2)-enhanced green fluorescent protein (EGFP) (Clontech Laboratories) into pAdeno-X expression system (Clontech Laboratories). First, we subcloned the human c-Maf cDNA (he-Maf) from its original vector into the EcoRI and SmaI sites in pIRES-EGFP, then transferred the he-Maf-IRES-EGFP fragment (SacI blunt and XbaI) into pShuttle vector (NheI blunt and XbaI). Finally, the human c-Maf and EGFP coexpression cassette (he-Maf-IRES-EGFP) was ligated with the pAdeno-X backbone by 1-EcoT and PI-SceI digestion. At the same time, we constructed an EGFP alone expression vector as a control by subcloning the IRES-EGFP sequence into pShuttle vector by NheI and NotI digestion, then transferred the EGFP alone expression cassette into pAdeno-X using the same construction strategy as described for the he-Maf-IRES-EGFP vector. Viruses were propagated in the human embryonic kidney 293 cell line and purified by ultracentrifugation through two cesium chloride gradients. Titers of viral stocks were determined by plaque assay in human embryonic kidney 293 cells after exposure to virus for 1 h in serum-free DMEM. Freshly isolated human monocytes were cultured in human M-CSF for 2 days. The resulting macrophages were exposed to recombinant virus (200 PFU/cell) for 4–8 h in serum-free RPMI 1640 medium followed by equal volume of RPMI 1640 supplemented with 10% FCS for an additional 24 h. The transfection medium was replaced with fresh RPMI 1640 medium with 10% FCS. Twenty-four hours later, the transfection efficiency was monitored by β-galactosidase staining (16) or EGFP expression under reverse-phase fluorescence microscope.

To construct the lacZ-based adenovirus vectors, Clontech Adeno-X Expression system (Clontech Laboratories) was used. We subcloned human c-Maf into the pShuttle vector, then subcloned the human c-Maf expression cassette into Adeno-X Viral DNA (Clontech Laboratories). Ad/ lacZ was constructed by subcloning lacZ expression cassette from pShuttle/lacZ (supplied in the Clontech Adeno-X Expression system as a positive control) into Adeno-X Viral DNA.

Transfections

Transient transfections were performed by electroporation as previously described (11). Transfection efficiency was routinely monitored by β-galactosidase assay by cotransfection with 3 μg of pCMV-β-galactosidase plasmid. Variability between samples was typically <10%. Lysates were used for both luciferase and β-galactosidase assays.

Cytokine assays

Cytokine secretion was measured by ELISA, using appropriately diluted culture supernatants. Human IL-12 p40 and p70, mouse IL-12 p40, p70, and IL-10 were measured by the respective ELISA kits from BD PharMingen (San Diego, CA).

RNase protection assay (RPA)

RPAs were performed using the human CK2b RibonQuant Multiprobe RPA system from BD PharMingen according to the manufacturer’s instructions. A total of 10 μg of RNA was used for each determination. The riboprobe for c-Maf was generated by transcribing c-Maf cDNA from pCEFL into PCR2.1 (Invitrogen, Carlsbad, CA) and linearization with NotI. The riboprobe was supplied in the Clontech Adeno-X Expression system as a positive control into Adeno-X Viral DNA.

RT-PCR

Reverse transcription (RT) reactions were conducted as follows: 0.4 μg total RNA was mixed with 2 μl oligo(dT) primers (16 mer, 0.5 mg/ml) and ddH2O to equalize volumes of all samples at 8.5 μl. The mix was boiled for 5 min, quenched on ice, spun down briefly, and 11.5 μl of a Master Mix was added. The RT Master Mix consisted of 4 μl 5 × first strand buffer (Life Technologies, Grand Island, NY), 4 μl 2.5 mM dNTPs, 2 μl 0.1 M DTT, 0.5 μl RNase inhibitor (40 U/μl; Boehringer-Mannheim, Indianapolis, IN), and 1 μl Superscript II RT (200 U/μl; Life Technologies). The reaction was incubated at 37°C for 90 min, then 95°C for 10 min, followed by a 4°C soak. To each sample (in 20 μl total volume) 80 μl ddH2O were added. A total of 2.5 μl were used for each PCR of 25 μl.

The following primers were used for PCR amplification: 1) CCR2 (U03905), upper: CACAGGGCGTCTATACATCAG, lower: CGACCTTATGATGCGTTCTTC; 2) glutaredoxin (X76648), upper: GCCCAAGAG ATCTCTCGACTA, lower: CAATTGGGCTTGTCTAAGCCT; 3) heme oxygenase (Ho-1) (X06985), upper: ATGACCAAGGACAGACAG, lower: TCAGTCCAGTCGCAAGAGG; 4) IL-1R type 2 (X50770), upper: TGCTTCTACGTCCTCCACTCT, lower: TACCCAGAGGCTGGTAC AAGG; 5) c-Jun N-terminal kinase 2 (L31951), upper: CCGTCCTTTTTC, lower: GCCCAAGAG ATCTCTCGACTA; 6) glutaredoxin (X76648), upper: GCCCAAGAG ATCTCTCGACTA, lower: CAATTGGGCTTGTCTAAGCCT; 7) hypoxanthine phosphoribosyltransferase (M26434.1), upper: CCTGCTGTGATACATCAAAGACT, lower: TC CAACACTTCTGTCGGTCTC.
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The thermal cycling conditions were 94°C for 3 min, 60°C for 30 s, and 72°C for 30 s for 1 cycle, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Nuclear extraction

Nuclear extractions for Western blot analysis and for EMSA assays were done according the method of Schreiber et al. (17). Briefly, 5–10 × 10⁵ cells were washed and resuspended in 600 μl of buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF for 15 min on ice. Cells were lysed in 0.6% Nonidet P-40 with vortexing for 10 s. The homogenate was centrifuged for 30 s in a microfuge and the nuclear pellet was resuspended in ice-cold buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF at 4°C for 15 min with rocking. Following centrifugation in a microfuge for 5 min, the supernatant was either used immediately or frozen at –70°C.

EMSA

EMSA and supershifts were performed as described previously (18). Oligonucleotides used for EMSA: ets-2, CCCAAAAGTCATTTCCTCTT AGTTCAATTA; GA-12, CCTCGTTATTTGATACACACAGAGA; NF-κB, ACTTCTTGAAATTCCCCCAGAAGG; and C/EBP/AP-1, GAGAG TTGTTTTCAATGTTGCAACAAGTCAGTTTCT. The underlined sequences are the respective binding motifs.

Generation of macrophages from the fetal liver of c-Maf knockout mice and genotyping

Disruption of both copies of the c-Maf gene affected both intrauterine and postnatal survival, so we derived macrophages of from day-14 embryos of c-Maf−/− mothers on a mixed background of 129 and C57BL6 (7). Mother mice were euthanized by CO₂ inhalation, the trunk soaked in 70% ethanol for 3–5 min, a midline incision was made on abdomen, and the gestational uterus was dissected exposing the embryos. Embryos were dissected and embryonic liver excised, then transferred into a 60-mm petri dish. A few drops of PBS were added to the liver, which was cut into small pieces with scissors. Single cells were prepared by mechanical disaggregating grinded with a syringe insert against a cell strainer (70 °C) and filtered through a syringe needle. Further single cells were prepared by mechanical disaggregating grinded with a syringe insert against a cell strainer (70 °C) and filtered through a syringe needle.

Western blotting

Western blot was performed as previously described (18).

Statistical analysis

Student’s t test was used for data analysis where appropriate. Data are expressed as mean ± SD unless otherwise indicated.

Results

c-Maf gene expression is induced by IL-10 and IL-4 in activated human peripheral blood monocytes

To identify some of the genes that are induced by IL-10 in monocytes activated by LPS or IFN-γ plus LPS, we prepared total RNA samples from human peripheral blood-derived monocytes purified by leukopheresis, labeled them with [α-³²P]dATP, and hybridized them to the Atlas Array (Clontech Laboratories) containing 1176 sequences as the respective binding motifs.

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FIGURE 1. Differential gene expression in activated human monocytes treated with IL-10. Elutriated human monocytes were stimulated with LPS (1 μg/ml) for 4 h or pretreated with recombinant human IL-10 (20 ng/ml) for 2 h followed by LPS stimulation. Some monocytes were pretreated with IFN-γ (10 ng/ml) for 16 h followed by IL-10 and LPS treatment. a and b, c-Maf protein expression in the nucleus. Human mature macrophages were replated after counting for further experimentation. Determination of the genotype of each embryo was performed by PCR using genomic DNA derived from a hind leg and primers that were able to differentiate the wild-type c-Maf gene from the disrupted copy (7).

Western blotting

Western blot was performed as previously described (18).

Statistical analysis

Student’s t test was used for data analysis where appropriate. Data are expressed as mean ± SD unless otherwise indicated.

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group of genes that were induced >4-fold in monocytes from five of five donors treated with LPS or IFN-γ plus LPS and IL-10 are listed in Table I. Several of these genes have been implicated in anti-inflammatory or Th2 responses (20–26). Among these, c-Maf caught our attention because of its role in promoting IL-4 gene expression and Th2 development, a process that functionally opposes IL-12-induced Th1 differentiation (10).

We verified by RT-PCR the status of differential expression of some of the genes identified in this search using RNA isolated from monocytes of a separate donor with the same stimulations. All genes appeared to be constitutively expressed, and the constitutive expression was inhibited in monocytes activated by LPS or IFN-γ plus LPS. Treatment of activated cells with IL-10 significantly up-regulated the mRNA expression of these genes (data not shown). The differential expression of c-Maf mRNA was confirmed by RPA, which revealed an inverse relationship with IL-12 (Fig. 1r). Notably, c-Maf mRNA expression in monocytes was also constitutive (lane 1). LPS or IFN-γ plus LPS challenge of monocytes resulted in its suppressed expression (lanes 2 and 5). Both IL-10 and IL-4 treatment of LPS-stimulated monocytes caused an induction of c-Maf mRNA (lanes 3 and 4), but IL-4 failed to induce c-Maf in IFN-γ/LPS-treated cells, and to inhibit IL-12 p35 and p40 expression (lane 7), suggesting that the mechanisms of induction of c-Maf expression by IL-10 and IL-4 are likely different.

Western blot analyses were also performed to evaluate the regulation of nuclear c-Maf protein production by IL-10 in human monocytes and mouse peritoneal macrophages. In human monocytes, c-Maf was constitutively present in the nucleus (Fig. 1d, lane 1). Upon LPS or IFN-γ plus LPS stimulation, c-Maf level was strongly reduced (lanes 2 and 4), whereas IL-10 treatment reversed this inhibition (lanes 3 and 5). This result is consistent with the mRNA data presented in Fig. 1d. In thioglycolate-elicited mouse peritoneal macrophages, c-Maf protein expression was also constitutive (Fig. 1e, lane 1). However, cellular activation by IFN-γ and LPS treatment did not result in a complete down-regulation of c-Maf (lane 2). IL-10 treatment for 1 or 2 h led to a strong up-regulation of c-Maf in these cells (lanes 3 and 4).

c-Maf expression in primary macrophages selectively inhibits IL-12 gene expression and induces IL-10 expression

To determine whether c-Maf expression in PBMC-derived human macrophages was causative for suppressed IL-12 production, we transduced human monocyte-derived macrophages obtained by culturing in M-CSF with an adenovirus carrying either a cDNA coding for human c-Maf or for the lacZ gene. As shown in Fig. 2a, macrophages transduced with a lacZ-expressing virus produced IL-12 p40 (upper panel) and p70 (middle panel) when stimulated with LPS alone (p40 only) or IFN-γ plus LPS. Culturing monocytes in M-CSF resulted in a “priming” effect for expression of IL-12 p40 but not p70, i.e., p40 production no longer depended on IFN-γ. Transduction of macrophages with the c-Maf-expressing virus caused a strong inhibition of IL-12 p40 and p70 secretion. However, IL-10 production was induced by c-Maf in resting macrophages, and markedly enhanced in LPS or IFN-γ plus LPS-stimulated cells (lower panel).

We next addressed the question of whether the inhibitory effects of c-Maf were restricted to IL-12. We transduced human macrophages with Ad/EGFP or Ad/c-Maf, and analyzed cytokine mRNA expression by RPA in these cells following appropriate stimulation

Table I. Genes that were significantly induced in LPS-activated human monocytes by IL-10

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Function or Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP-ribosylation factor I</td>
<td>M36340</td>
<td>Hydrolize GTP to GDP in vesicular transport</td>
</tr>
<tr>
<td>CCR2</td>
<td>U03905</td>
<td>Chemokine receptor induced by IL-10 in LPS macrophage</td>
</tr>
<tr>
<td>c-Maf</td>
<td>AF055377</td>
<td>Th2 transcription factor, protooncogene</td>
</tr>
<tr>
<td>Glutaredoxin</td>
<td>X76648</td>
<td>Carry electrons from NADPH to ribonucleotide reductase;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Important for protection against oxidative stress</td>
</tr>
<tr>
<td>HO-1</td>
<td>X06985</td>
<td>First step in degradation of heme to bilirubin and CO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(anti-inflammatory)</td>
</tr>
<tr>
<td>IL-1R type II</td>
<td>X59770</td>
<td>Mediate Th2 proliferation and cytokine production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Involved in NF-AT-dependent transcription, papilloma</td>
</tr>
<tr>
<td>JNK2b</td>
<td>L31951</td>
<td>Mediate IL10-dependent Th2 suppression in sepsis</td>
</tr>
<tr>
<td>MAPK p38</td>
<td>L35253</td>
<td></td>
</tr>
</tbody>
</table>

*The genes identified in this search are selected that satisfy all the following four criteria: 1) signal levels are >1000 in the IL-10-treated samples; 2) fold of change is >4 in IL-10-treated cells; 3) consistently induced in all five donors tested; 4) IL-12 production was inhibited in all IL-10-treated samples.

*JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.
with IFN-γ and LPS (Fig. 2b). c-Maf expression in these macrophages resulted in a strong inhibition of mRNA expression of IL-12 p40 and p35 with minimal effect on other cytokines and a strong induction of IL-10 mRNA synthesis, confirming the protein profiles of these cytokines (Fig. 2a). This indicates that c-Maf inhibits IL-12 gene expression selectively, and c-Maf and IL-10 could induce each other’s expression.

The observation of the ability of c-Maf to up-regulate IL-10 expression in inflammatory macrophages prompted the question of whether c-Maf-mediated inhibition of IL-12 production was dependent on IL-10. To address this possibility, we applied neutralizing IL-10 Ab to the cultures of c-Maf-transduced human macrophages. In two of three donors, the inhibition of IL-12 p40 or p70 production by the transduced c-Maf expression was not reversed at all by the presence of the Ab while there was a partial reversal in the third donor (data not shown). Nonetheless, in all three donors c-Maf transduction resulted in strong inhibition of IL-12 production. The lack of a consistent correlation between blocking of IL-10 in c-Maf-transduced macrophages and a reversal of c-Maf-mediated inhibition of IL-12 production led us to conclude that c-Maf does not inhibit IL-12 production simply by stimulating IL-10 production.

c-Maf differentially regulates IL-12 p35, IL-12 p40, and IL-10 gene transcription

To further delineate the molecular mechanism by which c-Maf exerts its inhibitory effects on IL-12 p40 and p35 transcription, we used a well-established transient transfection system in the murine monocytic cell line RAW264.7 (11). When the human IL-12 p40, p35, or IL-4 and IL-10 promoter-luciferase reporter constructs were cotransfected into RAW cells, IL-12 p40 and p35 promoter-driven luciferase activity in IFN-γ-LPS-stimulated cells was strongly inhibited by c-Maf expression (Fig. 3a). In contrast, IL-4 and IL-10 promoter activities were greatly enhanced, consistent with the reported selective role of c-Maf in IL-4 gene transcription (14). Notably, IFN-γ strongly suppressed c-Maf-induced IL-4 and IL-10 transcription.

It is generally believed that in transient transfections, the reporter gene (herein termed “transgene”) is not associated with a chromatin structure as is the endogenous gene. To ascertain if the observed effects of c-Maf on the IL-12 p40 reporter gene in RAW cells could also pertain to the endogenous (chromosomal) IL-12 p40 gene, we cotransfected the human IL-12 p40 promoter-reporter construct with the control vector pCEFL or the two isoforms of c-Maf (long and short; Ref. 5) in two amounts (reporter to effector molar ratio of 3:1 and 9:1, respectively). Both IL-12 p40 promoter-driven luciferase activity and the endogenous IL-12 p40 protein secretion in RAW cells stimulated by IFN-γ and LPS were strongly inhibited as a result of c-Maf expression (Fig. 3b), indicating that the transcription of IL-12 p40 is suppressed by c-Maf acting on the “episomal” and chromosomal genes alike.

Localization of the c-Maf-responsive element within the IL-12 p40 promoter

The human IL-12 p40 promoter contains three critical cis-elements involved in the regulation of its transcription by LPS and IFN-γ: an ets site at −211/−206 (TTTCTT), an “NF-κB half site” at −117/−107 (TGAAAATTC), and a C/EBP site at −72/−80 (ATGTTGCGA). The ets site and its surrounding sequences interacts with a large complex named F1, which is induced by either LPS or IFN-γ, and is composed of ets-2, PU.1, IFN regulatory factor-1, IFN consensus binding protein, NF-κB c-Rel, and a novel ets-2-related protein (11, 12, 27). The NF-κB half site binds p50/p65 and p50/c-Rel heterodimers induced by LPS (12, 28–30).

C/EBP site interacts with members of the C/EBP family, particularly C/EBPβ (31). Another motif recently described as a negative element in the IL-12 p40 promoter is the GA-12 site (GATA) located at −157/−160. The binding activity of the GA-12 binding protein GAP-12 was increased by treatment with two inhibitors of IL-12 expression, IL-4 and PGE2 in human CD14+ monocytes. Moreover, IL-4-mediated repression of IL-12 p40 promoter activity was critically dependent on an intact GA-12 sequence (32).

To identify the promoter element(s) through which c-Maf mediates its inhibitory effects on IL-12 p40 transcription, we used various deletion mutants of the p40 promoter-luciferase constructs, and cotransfected them with the c-Maf expression vector or the control vector into RAW264.7 cells. As shown in Fig. 4a, deletion of the 3300-bp p40 promoter to −222 reduced the overall promoter activities under the three experimental conditions but did not
affect the c-Maf-mediated inhibition of the IFN-γ/LPS-induced transcription. Further deletion of the promoter to -122, which eliminated the ets (11) and GA-12 (32) sites but retained the NF-κB site, drastically reduced the overall promoter activity (note the different scales of luciferase activity used), as reported previously (11), but did not block the response to c-Maf inhibition of IFN-γ/LPS-induced reporter activity. Removal of the NF-κB site by deleting a further 17 bp down to -105 resulted in almost complete loss of the inducibility of the promoter by IFN-γ and LPS, making it difficult to assess the ability of c-Maf to suppress its activity. Thus, the putative c-Maf-responsive element is either overlapping with the NF-κB site or located further downstream. Of note, the -122 and -105 constructs, as well as a construct that contains only the TATA box (data not shown), consistently responded positively to c-Maf expression in unstimulated or LPS-stimulated cells. This reinforces the notion that c-Maf may also act as a transcriptional activator, depending on the promoter context.

Because of the noted role of the C/EBP (31) and AP-1 (33) sites in the regulation of the mouse IL-12 p40 promoter, we sought to determine whether they were important for c-Maf-mediated inhibition of the human IL-12 p40 transcriptional induction. Base substitutions were introduced into these two sites separately by site-directed mutagenesis in the context of the -292/+108 IL-12 p40-luc construct. Cotransfection of these constructs with c-Maf was performed (Fig. 4b). Mutation of the C/EBP site resulted in a substantial reduction of the human IL-12 p40 promoter activity induced by IFN-γ/LPS, confirming the previously reported finding (31). However, c-Maf expression still caused a significant inhibition of the mutant promoter activity. In contrast, the AP-1 mutant did not affect the IL-12 p40 transcription (see Discussion for an explanation), nor did it affect c-Maf’s ability to suppress the induced p40 promoter activity. Taken together, we conclude that c-Maf’s inhibitory effects on IL-12 p40 promoter activation are not likely mediated through these two sites.

Role of the NF-κB site in c-Maf-mediated inhibition of IL-12 p40 transcription

Next, we focused more closely on the NF-κB site. We cotransfected the -222 wild-type construct with the c-Maf expression vector, and compared the luciferase activity to that of a mutant

FIGURE 4. Localization of the c-Maf-responsive element in the human IL-12 p40 promoter. a, A series of 5’ deletion mutants of the full-length human IL-12 p40 promoter-luciferase construct, which spans 3.3 kb upstream and 108 bp downstream of the transcription initiation site, were cotransfected transiently with a c-Maf expression vector or the control vector pCEFL into RAW264.7 cells at a molar ration of 3:1 (reporter:effector). Cells were then stimulated with LPS alone or IFN-γ (16 h) followed by LPS (7 h). Cell lysates were assayed for luciferase activity. The data are summaries of four separate experiments. b, Effect of mutations at the C/EBP and AP-1 sites. Mutant promoter luciferase constructs of IL-12 p40 promoter in the context of -292/+108 were cotransfected with c-Maf at a molar ratio of 1:1. Luciferase activity was measured from cell lysates following stimulation of RAW264.7 cells with IFN-γ and LPS, and normalized to the medium condition of the wild-type IL-12 p40 construct, which was taken as 1. Data represent mean plus SD of three independent experiments with duplicate measurements each.
A functional c-Maf consists of an N-terminal transactivation domain, a central, basic DNA-binding domain, and a C-terminal leucine zipper dimerization domain. To determine the requirement of these domains in the inhibition of IL-12 p40 transcription, we made two constructs of c-Maf with sequential deletions from the N terminus such that it contained no transactivation domain, but retained the DNA-binding and LZs (basic-LZ), or one that contained the LZ only. The ability of these deletion constructs of c-Maf to suppress IL-12 p40 transcription and to activate IL-10 promoter was tested by transient transfection assay in RAW264.7 cells. As shown in Fig. 7a, neither mutant construct was able to inhibit IL-12 p40 transcription stimulated by IFN and LPS or activate IL-10 transcription induced by LPS to the degree attained by the full-length construct despite more or less equivalent expression levels of their respective proteins in the nucleus (Fig. 7b), suggesting that the N-terminal transactivation domain is required for c-Maf to play its negative and positive role for IL-12 p40 and IL-10 transcription, respectively.

IL-10 production in c-Maf-deficient macrophages is impaired, whereas IL-10-mediated inhibition of IL-12 p40 production is intact

An important question was whether c-Maf is the sole mediator of IL-10’s inhibitory effects on IL-12 production by macrophages. To address this issue, we obtained c-Maf-deficient murine macrophages derived from the fetal liver of day-14 embryos because of the prevalent embryonic lethality of homozygous c-Maf deficiency (7). In c-maf-deficient macrophages derived from fetal liver (one wild type, two heterozygotes, and six homozygotes), the levels of IL-12 p40 production induced by LPS or IFN-γ plus LPS were comparable in the three groups while IL-10 production was impaired in c-Maf−/− macrophages (Fig. 8a). However, IL-10 treatment of LPS- or IFN-γ/LPS-activated macrophages strongly suppressed IL-12 p40 production, displaying no discernible difference from the normal or heterozygous macrophages (Fig. 8b).
Discussion
In this study, we took a genome-wide approach to search for genes that are induced by IL-10 in pathogen/cytokine-activated human monocytes with a further objective to identify those that are involved in the inhibition of IL-12 production.

The observation that all of the genes identified by this approach including c-Maf are constitutively expressed and that IL-10 treatment merely reverses their inhibition by macrophage-activating agents suggests that IL-10’s general function may be to maintain a homeostasis of cellular activities. In other words, the intrinsic activities of IL-10 are to bring an activation state back to a resting state in a reactionary manner, as opposed to a "proactive" function in which IL-10 would vigorously seek its own agenda.

We also demonstrated for the first time that IL-10 and c-Maf are capable of enhancing each other’s expression, forming a positive amplification loop that is likely to reinforce one another’s activities, leading to a profound impact.

We have partially localized the c-Maf-responsive element within the IL-12 p40 promoter by a reductionist approach of deletions and mutations to a region downstream of the C/EBP and AP-1 site (Fig. 4), but upstream of +20 (data not shown). Our AP-1 mutant did not result in any reduction in the p40 promoter activity induced by IFN-γ and LPS, a result that differs from that of Zhu et al. (33). The reason for this discrepancy is not immediately clear. Possibly, species differences could account for such a discrepancy in that the human IL-12 p40 promoter-luciferase
reporter was used in our study and the study in which the role of the AP-1 site was investigated used the murine IL-12 p40 promoter linked to a CAT reporter.

Analyses of direct nuclear DNA-binding activities at the critical sites such as ets, GA-12, NF-H9260B, and C/EBP/AP-1 elements showed significant changes induced by c-Maf expression (Fig. 6). However, these changes do not seem to be essential for c-Maf-mediated inhibition of p40 transcription as removal of promoter regions harboring these sites or site-directed mutagenesis in some of these elements did not impact on c-Maf’s ability to inhibit the p40 promoter activity. Thus, these sites targeted by c-Maf are redundant with respect to c-Maf-mediated inhibition, and the precise

FIGURE 7. Inhibition of IL-12 p40 transcription and activation of IL-10 transcription by c-Maf requires its N-terminal transactivation domain. a, Transient cotransfections were performed as described in Fig. 5 using IL-12 p40 or IL-10-luciferase reporters with the wild-type c-Maf expression vector or its deletion derivatives. The control was IFN-γ/LPS- (for IL-12 p40) and LPS-stimulated cells (for IL-10), which maximize their respective promoter activation (see Fig. 3a). Luciferase activities are expressed as percentage of that of the control. FL, full-length c-Maf; basic-LZ, c-Maf construct containing no trans-activation domain but retaining the DNA-binding (basic) and dimerization (LZ) domains; LZ, c-Maf construct containing only the protein dimerization domain. Some basic structural features of the wild-type c-Maf are indicated. b, Nuclear protein expression of the transfected c-Maf constructs was analyzed by Western blot using a flag Ab (hemagglutinin-tag). Note that the nuclear extracts were derived from RAW264.7 cells stimulated with IFN-γ and LPS. Data are representative of three independent experiments.

FIGURE 8. IL-10 production is impaired in c-Maf-deficient macrophages, whereas IL-12 p40 production and its inhibition by IL-10 are intact. Macrophages were derived from fetal liver extracted from day 14 embryos of c-Maf heterozygous (+/−) pregnant mothers; their genotypes were determined by PCR as described in Materials and Methods. Cells were plated at 1 × 10⁶/well, stimulated with LPS or IFN-γ plus LPS in the presence or absence of IL-10 (10 ng/ml). Twenty-four hours following stimulation, cells were harvested and cell-free supernatant assayed for mIL-12 p40 and mIL-10 production by ELISA. a, IL-12 p40 and IL-10 production following stimulation. Data are derived from one wild-type, two heterozygous, and six homozygous embryos with SEM. b, IL-10 treatment-induced inhibition of IL-12 p40 production in IFN-γ plus LPS-stimulated macrophages. This separate experiment involved one wild-type (+/+), two heterozygous (+/−), and five homozygous (−/−) embryos. The lower limits of the ELISA were 15.6 pg/ml for mIL-12 p40 and 31 pg/ml for mIL-10. bdr, below detection range.
location of the essential putative c-Maf-response element remains elusive.

The requirement of the N-terminal domain of c-Maf for its IL-12-inhibiting activity (Fig. 7a) has two implications. The first is that c-Maf may act directly on the p40 promoter in conjunction with an additional factor producing a repressive outcome. This is unlikely since we were unable to identify a site on the p40 promoter to which c-Maf or its associated forms bind (data not shown). The second possibility is that the inhibitory effect of c-Maf may be mediated through an intermediary, i.e., c-Maf induces another factor which in turn suppresses IL-12 p40 transcription. A precedent of such an indirect repression mechanism by c-Maf was reported by Hegde et al. (34), who showed that expression of c-Maf in human immature myeloblastic cells inhibited the transcription of the myeloid lineage-restricted CD13/APN gene by inducing the binding of both c-Myb and ets-1 to the promoter without its own direct interaction with the target gene. In a preliminary experiment to identify this putative intermediate factor using the Affymetrix microarray containing 12,600 genes and comparing mRNA expression profiles of Ad/c-Maf-transduced human macrophages vs Ad/GFP-transduced cells, four nuclear factors were found to be significantly induced by c-Maf expression (≈2-fold). Interesting and relevant among these is the protein inhibitor of activated Stat1 (PIASx-α), which inhibits Stat1-mediated gene activation (35). The uncovering of this transcriptional repressor in c-Maf-mediated inhibition of IL-12 gene expression is most likely related to the fact that our cells had been treated with IFN-γ, which induces Stat1. Further investigation is in progress.

The preferential inhibition of IL-12 gene transcription by c-Maf is not surprising given the previous demonstration of its highly selective activation of IL-4 transcription without affecting the expression of many other Th2 cytokines (10). Our observation that TNF-α expression is not regulated by c-Maf (data not shown) suggests that other mediators of IL-10’s broad anti-inflammatory activities exist that operate independently of c-Maf to modulate inflammation. Some of the other genes identified in our limited search (summarized in Table I) likely play a role in the multiple regulatory pathways controlled by IL-10. For example, the stress-inducible protein HO-1 provides protection against oxidative stress. This anti-inflammatory activity of HO-1 is mediated by carbon monoxide, a by-product of heme catabolism by HO. Furthermore, carbon monoxide mediates its anti-inflammatory effects, including the inhibition of LPS-induced expression of TNF-α, IL-1β, and macrophage-inflammatory protein-1β and enhancement of IL-10 production through a pathway involving the p38 MAPK (36).

Members of the Maf family of bZIP transcription factors can affect transcription in either a positive or negative fashion, depending on their particular protein partner and the context of the target promoter (14, 34, 37–45). This functional duality of c-Maf is consistent with our observation that c-Maf can switch from being a transcriptional repressor of the IL-12 p40 promoter containing sequences extending 5' beyond the NF-xB site at -115, to an activator of the p40 promoter which is deleted down to the NF-xB site or further downstream (Fig. 4). The underlying mechanism of this specific switch is not presently understood. It may involve dynamic interactions between c-Maf-induced complexes and pathway/cytokine-activated transcription factors which bind to sites upstream of the NF-xB element in the context of preassembled basal transcription machinery.

The apparent dispensability of c-Maf in IL-10-mediated inhibition of IL-12 production in fetal liver-generated macrophages (Fig. 8b) indicates a functional redundancy in this pathway. We have preliminary evidence indicating that that endogenous AP-1 is a transcriptional inhibitor of the IL-12 p40 gene, and IL-10’s inhibition of IL-12 p40 protein synthesis is dependent, at least in part, on AP-1 (our published data), suggesting that AP-1 could be such an alternative mediator. In support of this supposition, it is noted that p38 MAPK, an essential activator of AP-1, is induced by IL-10 in activated human macrophages (Table I). In ovarian and endometrial carcinoma cells, IL-10 directly stimulates AP-1 activity (46).

Alternatively, the IL-10 dependency of c-Maf for its transcription repression of IL-12 p40 may be different between mouse and human, or between liver macrophages and those from other anatomical compartments such as spleen and peritoneal cavity. Presently, technical difficulties preclude direct demonstration and discerning of these possibilities.

In summary, we have established a novel role of c-Maf in the selective and opposing regulation of IL-10 and IL-12 gene transcription in macrophages. Yet, c-Maf is apparently a redundant mediator of IL-10’s suppressive activity on IL-12 production. The implications are 2-fold. It suggests the possibility that c-Maf, as an immunological regulator, could play a dual role of driving humoral immunity via stimulation of IL-4 transcription, and suppressing innate immune responses by inhibiting IL-12 production. It also implies that the other property of c-Maf, its oncogenic potential, could be exerted through its intimate interaction with the immune surveillance of the host and manifested in the form of selective inhibition of such critical activators of tumor-busting CTL and NK cells as IL-12.

References


