

Activating transcription factor 3 (ATF3) represses the expression of CCL4 in murine macrophages

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Abstract

Acute expression of macrophage inflammatory protein-1 beta (also known as CCL4) promotes beneficial leukocyte recruitment to infected tissues, but chronic expression of this chemokine contributes to inflammatory disease. CCL4 expression is controlled largely at the transcriptional level and an ATF/CRE sequence located in the promoter (−104 to −97 bp, relative to the transcriptional start site) has been identified as a critical *cis*-acting element. The *trans*-acting binding proteins that influence CCL4 transcription via this site are largely unknown. We investigated whether activating transcription factor 3 (ATF3), a member of the ATF/CREB family of transcription factors, binds to the CCL4 ATF/CRE site in macrophages. Using the electrophoretic mobility shift assay and the chromatin immunoprecipitation assay, we found that ATF3 binds to the ATF/CRE site within the CCL4 promoter in untreated and lipopolysaccharide (LPS)-stimulated macrophages. Quantitative RT-PCR analysis showed that CCL4 mRNA levels in elicited peritoneal macrophages from ATF3^{−/−} mice are significantly higher than in congenic ATF3^{+/+} macrophages under both unstimulated and LPS-stimulated conditions, suggesting that ATF3 represses transcription of the CCL4 gene. Consistent with the higher gene expression, ATF3-deficient macrophages secreted more CCL4 protein than ATF3^{+/+} macrophages. Similar results were obtained in bone-marrow-derived macrophages treated with Toll-like receptor 2, 3, 4 and 5 agonists. Thus, we conclude that ATF3 constitutively binds to the ATF/CRE site in the CCL4 promoter where it represses basal and pathogen-associated molecular pattern (PAMP)-stimulated transcription. Consequently, ATF3 appears to be part of a control mechanism that limits the amount of CCL4 released by macrophages, preventing excessive inflammation.

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1. Introduction

Macrophage inflammatory protein-1 beta (MIP-1β) also known as CCL4 (Menten et al., 2002) is an 8 kDa chemokine that was originally isolated from the medium of bacterial lipopolysaccharide (LPS)-activated macrophages (Wolpe et al., 1988). Though macrophages are major producers of CCL4, dendritic cells, lymphocytes and other cells also secrete this chemokine (Menten et al., 2002). CCL4 mediates inflammatory responses by stimulating the chemotaxis of monocytes, immature dendritic cells, Th1 lymphocytes, NK cells, and activated eosinophils (Broxmeyer et al., 1990). The expression of CCL4 is induced in response to bacterial and viral infection and in at least some cases this chemokine is vital for effective clearance

of the microbes. However, elevated levels of CCL4 are found in the most affected tissues in several chronic inflammatory diseases (Menten et al., 2002). Consequently, it is believed that CCL4 contributes to detrimental chronic inflammatory conditions in addition to having beneficial actions in acute inflammatory responses.

The regulation of CCL4 expression in immune system cells has not been well studied, although there is some published work on macrophage lineage cells. These reports reveal that CCL4 mRNA and protein levels are very low in resting human and murine monocyte/macrophages (Proffitt et al., 1995; Widmer et al., 1993; Wolpe et al., 1988; Ziegler et al., 1991). Upon exposure to microbial stimuli such as LPS, CCL4 mRNA levels are induced within 30 min, reach maximum levels by 4–6 h, and then decline thereafter. The increased mRNA level is primarily due to transcriptional induction (Ziegler et al., 1991). However, stabilization of the mRNA is another potential site of regulation, since the 3′ untranslated region contains an AU-rich sequence

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(Widmer et al., 1993) found in the mRNAs of many cytokines controlled in this manner (Tebo et al., 2003). Deletion analysis of the CCL4 promoter has identified a LPS-responsive element located –127 to –95 relative to the transcriptional start site (Proffitt et al., 1995; Ziegler et al., 1991). Within this region is a putative ATF/CRE element [TGACATCA] conserved in the human and mouse CCL4 promoters that is necessary for the LPS responsiveness; nucleotide substitution within the ATF/CRE sequence [gGAtATCg] substantially reduces the induction by LPS (Proffitt et al., 1995). Thus, the putative ATF/CRE element is a focal point for transcriptional regulation of CCL4 expression in LPS-treated macrophages.

Several different protein complexes bind to the ATF/CRE site in the CCL4 promoter, based on the multi-band electrophoretic mobility shift assay (EMSA) pattern observed by Proffitt et al. (1995). To date, c-Jun is the only protein that has been shown to bind to this element. However, the functional relevance of c-Jun binding to the regulation of CCL4 transcription has not been demonstrated. Importantly, the published EMSA data illustrating c-Jun binding showed multiple protein–DNA complexes not supershifted by c-Jun antibodies (Proffitt et al., 1995), suggesting that proteins other than c-Jun bind to the ATF/CRE site.

The ATF/CRE in the CCL4 promoter has the same sequence as the NF-ELAM1 element in the E-selectin promoter (TGACATCA) (Hooft van Huijsduijnen et al., 1992). Since NF-ELAM1 has been shown to bind ATF3 (Kaszubska et al., 1993; Nawa et al., 2000), a member of the ATF/CREB family of transcription factors (Hai and Hartman, 2001), we investigated whether ATF3 binds to the ATF/CRE in the CCL4 promoter. In this report, we show that ATF3 binds to the ATF/CRE site in the CCL4 promoter and represses basal and LPS-stimulated transcription of CCL4. Thus, ATF3 may curtail the amount of CCL4 secreted by macrophages, thereby preventing damaging inflammatory responses.

2. Materials and methods

2.1. RAW264.7 cell culture

The murine macrophage cell line, RAW264.7 (American Tissue Type Collection, Rockville, MD) was cultured as described (Weinstein et al., 1991). For preparation of nuclear extracts, 3×10^7 cells in 20 ml of medium were seeded onto 15 cm dishes (Corning Inc., Acton, MA) and incubated at 37 °C, for 12–16 h. Highly purified LPS from *Salmonella minnesota* R595 (Re) (List Biological Laboratories, Campbell, CA) was added to a final concentration of 100 ng/ml and the cultures were incubated at 37 °C for 1 h. The cells were lysed and nuclear extracts were prepared as described in (Kitchens et al., 1992).

2.2. Primary macrophage cultures

Thioglycollate-elicited peritoneal macrophages were prepared from 6- to 8-week-old ATF3-deficient (Hartman et al., 2004) and C57Bl/6 mice as described in (Gallily and Feldman, 1967). Peritoneal washout cells from several mice were pooled, plated at 2×10^6 cells in 4 ml of α -MEM containing 10% heat-

inactivated fetal bovine serum, and 50 μ g/ml gentamycin in 6-well plates (Corning, Acton, MA) and incubated at 37 °C for 2 h. Non-adherent cells were removed by washing the cultures with PBS and the resultant adherent macrophage cultures were incubated in fresh α -MEM medium overnight. Prior to the addition of LPS, the medium on the cultures was replaced with 1.5 ml of fresh α -MEM medium. Following the stimulation period, the cultures were washed twice with 2 ml of PBS and the cells were lysed in 350 μ l of RLT buffer (RNeasy kit, Qiagen, Valencia, CA). The resultant cell lysates were stored at –80 °C until further processing was performed.

Bone marrow cells were isolated from the femurs and tibias of the same mice used to prepare peritoneal macrophages. Bone marrow-derived macrophages were generated as described in (Yi and Wilman, 1989), except that the cells were cultured in α -MEM supplemented with 10% fetal bovine serum, 50 μ g/ml gentamycin and 20% CMG 12–14 cell conditioned medium as a source of CSF-1 (Takeshita et al., 2000). Non-adherent macrophage precursors (3×10^5) were seeded into 60 mm dishes in 2 ml of medium. These cultures were given fresh medium every third day and were used for experiments after 9 days. Macrophages were stimulated with LPS and lysed in RLT buffer as described for the peritoneal macrophages. The San Francisco State University Institutional Animal Care and Use Committee approved all animal protocols.

2.3. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed as described in (Buratowski and Chodosh, 1996). Nuclear extracts (5 μ g) were incubated (30 min at 25 °C) with a ³²P-labeled double-stranded oligonucleotide containing the ATF/CRE sequence (underlined) from the CCL4 promoter CTCGATGCCATGACATCATCTTTACTCA or a mutated ATF/CRE site CTCGATGCCAgGAtATCgTCTTTACTCA, that does not bind c-Jun (Proffitt et al., 1995). Some nuclear extracts were incubated with rabbit polyclonal antibodies (3 μ g) against ATF3 or c-Fos (sc-188 and sc-253, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 25 °C prior to incubation with the radiolabeled oligonucleotide. Protein–DNA complexes were resolved by electrophoresis on a 5% non-denaturing polyacrylamide gel and detected by autoradiography.

2.4. Quantitative RT-PCR

Total RNA was isolated from macrophage RLT lysates using an RNeasy kit with DNase I treatment according to the manufacturer's instructions. RNA was quantitated by absorbance at 260 nm and 800 ng of total RNA was used to synthesize cDNA using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed in triplicate for each sample with Taqman primer/probes on an Applied Biosystems 7900 system at the UCSF Comprehensive Cancer Center Genome Analysis Core. Each 20 μ l reaction contained 5 ng cDNA, 5.5 mM MgCl₂, 200 μ M dNTPs, and 1.25 units AmpliTaq Gold DNA polymerase. PCR was carried out for 40 cycles of 95 °C for

15 s and 60 °C for 1 min, after an initial 10 min incubation at 95 °C. The relative amount of CCL4 mRNA normalized to β -glucuronidase mRNA was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.5. Intracellular CCL4 staining

Bone marrow-derived macrophages cultured to 90–95% confluent on 60 mm dishes were stimulated with 100 ng/ml of highly purified LPS for 6 h. During the last 4 h of culture, Golgi Plug reagent (BD Biosciences, San Diego, CA) was added to block protein secretion. Following the stimulation period, the cells were released from the dishes by incubating in 5 mM EDTA/PBS. Cells were pelleted by centrifugation and washed twice with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS (Invitrogen, Carlsbad, CA) containing 1% FBS (Invitrogen, Carlsbad, CA) and 0.09% sodium azide. Surface Fc receptors were blocked by incubating the cells in 4% mouse serum (Jackson ImmunoResearch Lab, West Grove, PA) in PBS for 30 min on ice. The cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (San Diego, CA), followed by intracellular blocking with 5% horse serum in PBS for 30 min on ice. Cells (5×10^5) were then stained with 0.5 μg of a rat anti-mouse CCL4 monoclonal antibody (clone A65-2, BD Pharmingen, San Diego, CA) or with an isotype control rat anti-HA monoclonal antibody (Roche Applied Science, Indianapolis, IN) for 30 min on ice followed by incubation with 0.5 μg of APC-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Lab) for 30 min on ice. Stained cells were analyzed with a FACSCalibur using CellQuest Software (Becton Dickinson, San Jose, CA). The median fluorescence intensity (MFI) was calculated from the MFI of anti-CCL4-stained cells minus the MFI of anti-HA-stained cells.

2.6. CCL4 ELISA

Quantitation of CCL4 protein secreted into the culture medium was performed using a sandwich ELISA kit (DuoSet ELISA kit, R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

2.7. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed using an EZ CHIP kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol with some modification. Briefly, RAW264.7 cells (1×10^8 in 100 ml of medium) were plated on 150 cm^2 plates (Corning) and incubated overnight. The next day the cells were treated with 100 ng/ml of LPS for 60 min. Following LPS treatment, the plates were washed twice in PBS and the cells were incubated in 1% formaldehyde for 12.5 min at room temperature. Glycine was added to 125 mM final concentration to terminate the reaction. The plates were washed three times in PBS, then the cells were dislodged with a cell lifter and collected by centrifugation ($400 \times g$, 5 min, 4 °C). Lysis buffer [10 mM Tris pH 8, 0.25% Triton X-100, 0.5% NP-40 and protease inhibitor cocktail] was added and the cells were incubated on ice for 10 min. Following

centrifugation, the cell pellet was resuspended in 5 ml of 50 mM Tris pH 8, 1% SDS, 10 mM EDTA and protease inhibitor cocktail and the DNA was sheared using a Misonix model XL20202 sonicator (Farmingdale, NY) at setting 5 (9×20 s pulses). The sheared chromatin samples were clarified by centrifugation ($13,000 \times g$, 10 min at 4 °C) and the supernatant fractions were diluted 1:10 in 16.7 mM Tris-HCl pH 8, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA and 167 mM NaCl. Pre-clearing was performed with an overnight incubation with protein G Sepharose (240 μl of a 50% slurry). For immunoprecipitation, 10 μg of anti-CREB (06-863, Upstate Biotechnology) or anti-ATF3 antibodies were added and the samples were incubated overnight at 4 °C. The immune complexes were collected with protein G Sepharose, washed extensively and eluted in 500 μl of 0.1 M NaHCO_3 , 1% SDS. Crosslinking was reversed by adding 20 μl of 5 M NaCl and heating to 65 °C for 12 h. Proteinase K (20 μg), 10 μl of 0.5 M EDTA and 20 μl of 1 M Tris-HCl pH 6.5 were added to each eluate and the samples were incubated at 45 °C for 60 min. The samples were extracted twice in phenol/chloroform and then the DNA was precipitated in ethanol, using 20 μg of glycogen as a carrier. After washing the DNA pellet in 70% ethanol, the DNA was resuspended in 100 μl of water and 20 μl was used as template for PCR analysis. For detection of the CCL4 promoter, DNA oligonucleotide primers (5'-CCTCCTTTCTCTCCGTGATG-3' and 5'-GCCCAAGCCTGACTATTGAGC-3') specific for a 413-bp region encompassing the ATF/CRE in the proximal CCL4 promoter were used for the first stage of PCR amplification. Thirty cycles of amplification were performed using Taq polymerase (Promega, Madison, WI). The PCR product (3 μl) was used as a template for a second round of amplification using nested primers (5'-TTCAGTTTCTTTCTCGATGCC-3' and 5'-GAGAACCCTGGAGCACAGA-3') for 10 cycles. The presence of the 270-bp product was evaluated on a 1% agarose gel. Images were acquired using a digital camera (UVP Inc., Upland, CA). The identity of PCR products was confirmed by DNA sequencing.

2.8. Statistical analysis

Two group, unpaired *t*-test analysis was performed using StatView 5.0 software, SAS (Cary, NC). All *p*-values <0.05 were considered statistically significant.

3. Results

3.1. ATF3 binds to the ATF/CRE site in the CCL4 promoter

To test whether ATF3 binds to the ATF/CRE site in the CCL4 promoter, we performed EMSA (Kitchens et al., 1992) and supershift experiments using nuclear extracts from the RAW264.7 macrophage cell line. As shown in Fig. 1A, multiple bands appeared in the EMSA, consistent with the literature that many proteins bind to the ATF/CRE site derived from the CCL4 promoter. Significantly, a supershifted band that migrated at the top of the gel (indicated by the arrowhead) appeared in the presence of the anti-ATF3 antibodies (Fig. 1A, lanes 9 and

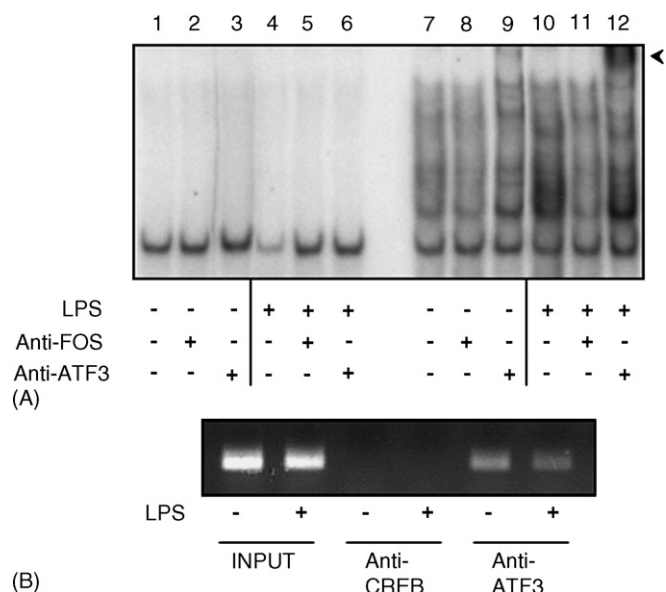


Fig. 1. ATF3 binds to the ATF/CRE site within the CCL4 promoter. (A) EMSA: nuclear extracts from unstimulated (–) and LPS-stimulated (+) RAW264.7 cells were incubated with a DNA fragment derived from the CCL4 promoter containing either a mutant (lanes 1–6) or wild type (lanes 7–12) ATF/CRE site. The reactions were carried out with or without prior incubation with the indicated antibodies and then analyzed by PAGE. The arrow on the right indicates the super-shifted band in the presence of anti-ATF3 antibodies. Similar results were obtained in four independent experiments. (B) ChIP: Sheared, cross-linked genomic DNA from unstimulated (–) and LPS-stimulated (+) RAW264.7 cells (input) was immunoprecipitated with anti-ATF3 or anti-CREB antibodies. The presence of the CCL4 promoter in the immunoprecipitated materials was assessed by nested PCR as described in the materials and methods. Shown are representative results from two experiments.

12), indicating the binding of ATF3 to the DNA. The specificity of ATF3 binding was confirmed by two controls. First, isotype-matched antibodies against Fos-family proteins (c-Fos, FosB, Fra-1 and Fra-2) did not cause a supershift comparable to the one produced by anti-ATF3 antibodies (Fig. 1A, lanes 8 and 11). Second, ATF3 binding was not detected when a mutant probe containing three nucleotide substitutions within the ATF/CRE site was used (Fig. 1A, lanes 3 and 6). We note that nuclear extracts from either untreated (Fig. 1A, lane 9) or LPS-stimulated (Fig. 1A, lane 12) RAW264.7 cells showed evidence of ATF3 binding to the CCL4 promoter. This is consistent with our ATF3 immunoblot analysis (data not shown), indicating that this protein is present in both untreated and LPS-treated cells.

To determine whether ATF3 binds to the CCL4 promoter *in vivo*, we performed a chromatin immunoprecipitation (ChIP) experiment. Soluble chromatin from unstimulated and LPS-treated RAW264.7 macrophages were immunoprecipitated with anti-ATF3 antibodies, then the presence of the CCL4 promoter in the immunoprecipitated fraction was evaluated by nested PCR. The expected 270-bp PCR product corresponding to bases –202 to +68 in the CCL4 promoter was detected in anti-ATF3 immunoprecipitates from both unstimulated and LPS-treated macrophages (Fig. 1B). In contrast, antibodies against another ATF/CREB family transcription factor, CREB, did not immunoprecipitate the CCL4 promoter. Taken together, the ChIP and

EMSA results indicate that ATF3 binds to the ATF/CRE site on the CCL4 promoter in macrophages.

3.2. CCL4 mRNA and protein are elevated in ATF3-deficient peritoneal macrophages

ATF3 regulates the transcription of a growing number of genes (reviewed in Hai et al., 1999). As a homodimer, ATF3 acts as a repressor; however, ATF3 has been shown to be a transactivator when dimerized with other basic region leucine zipper (bZip) proteins (Hai et al., 1999). Since our ChIP and EMSA data indicated that ATF3 binds to the CCL4 promoter, we asked whether ATF3 influences the transcription of the CCL4 gene. To this end, we compared the level of CCL4 mRNA in primary macrophages isolated from ATF3-deficient mice (ATF3^{–/–}) (Hartman et al., 2004) to that in ATF3^{+/+} mice. Thioglycollate-elicited peritoneal macrophages from ATF3^{–/–} and ATF3^{+/+} congenic C57Bl/6 mice were cultured in the presence or absence of LPS, and then the relative amount of CCL4 mRNA in these cells was determined by quantitative reverse transcription-real time PCR (qRT-PCR). As shown in Fig. 2A, we found that the basal, unstimulated level of CCL4 mRNA was significantly elevated (>13-fold) in ATF3^{–/–} macrophages compared to ATF3^{+/+} macrophages. Similarly, LPS-treated ATF3^{–/–} macrophages expressed significantly more CCL4 mRNA than ATF3^{+/+} macrophages (Fig. 2B). The disparity in CCL4 mRNA levels between ATF3^{–/–} and ATF3^{+/+} macrophages was the greatest at the earliest LPS stimulation times examined (three-fold more after 1 h with LPS and two-fold more after 2 h with LPS). Overall, the kinetics of LPS-induced CCL4 mRNA were comparable in ATF3^{+/+} and ATF3^{–/–} macrophages.

To determine whether the elevated CCL4 mRNA levels observed in ATF3^{–/–} macrophages led to higher CCL4 protein production, we compared the amount of CCL4 secreted by ATF3^{–/–} and ATF3^{+/+} peritoneal macrophages by ELISA before and after LPS treatment. We found that untreated ATF3^{–/–} macrophages secreted approximately 20-fold more CCL4 than ATF3^{+/+} peritoneal macrophages (601 ± 58 pg/ml versus 28 ± 2 pg/ml, mean ± S.E.M.) (Fig. 3). Following LPS-treatment, ATF3^{–/–} peritoneal macrophages also secreted significantly more CCL4 than ATF3^{+/+} cells at early treatment times (at 1 h: 889 ± 29 pg/ml versus 226 ± 16 pg/ml and at 2 h: 5.1 ± 0.2 ng/ml versus 3.4 ± 0.2 ng/ml). We note that, at later time points, the magnitude of the difference was smaller (data not shown). Thus, ATF3^{–/–} peritoneal macrophages produced more CCL4 protein than their ATF3^{+/+} counterparts, paralleling the elevated CCL4 mRNA levels found in ATF3-deficient macrophages.

3.3. CCL4 mRNA and protein are elevated in ATF3^{–/–} bone marrow-derived macrophages

To assess whether the effects of ATF3-deficiency on CCL4 levels seen in peritoneal macrophages occur in other populations of macrophages, we examined CCL4 mRNA expression in bone marrow-derived macrophages (BMDM ϕ) obtained from ATF3^{–/–} and ATF3^{+/+} mice. In these experiments, we treated the

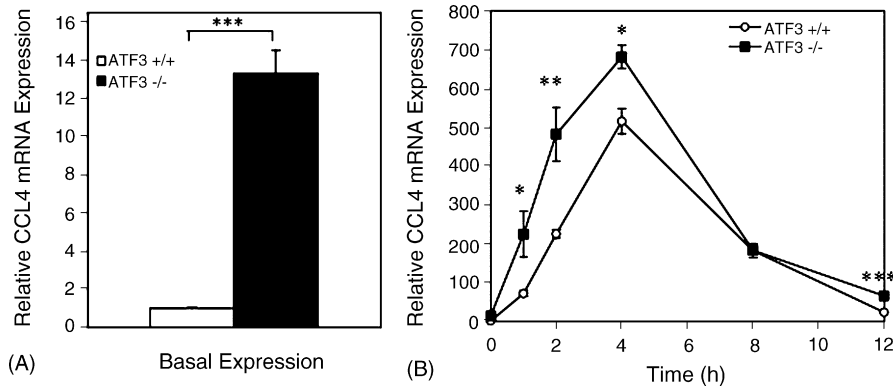


Fig. 2. Basal and LPS-stimulated CCL4 mRNA levels are higher in peritoneal macrophages from ATF3^{-/-} mice vs. ATF3^{+/+} mice. Thioglycollate-elicited macrophages from ATF3^{-/-} and ATF3^{+/+} mice were untreated (A) or exposed to 10 ng/ml of LPS for the indicated time (B). Total RNA was isolated and analyzed for CCL4 and β -glucuronidase mRNAs by qRT-PCR. The CCL4 expression in each sample relative to the unstimulated ATF3^{+/+} sample, normalized for equal β -glucuronidase expression was calculated by the $2^{-\Delta\Delta C_t}$ method. Shown are the mean \pm S.E.M. CCL4 expression determined from quadruplicate independent samples. *t*-Test *p*-values <0.05, <0.01 or <0.001 are indicated by *, ** or ***, respectively. Similar data were obtained in three separate experiments, each using a different group of mice.

macrophages with various reagents to stimulate different Toll-like receptors (TLRs): lipoteichoic acid (LTA) for TLR2, poly I:C for TLR3, LPS for TLR4, and flagellin for TLR5. We found that the basal amount of CCL4 mRNA in ATF3^{-/-} BMDM ϕ was elevated compared to ATF3^{+/+} BMDM ϕ (Fig. 4 inset). Following exposure to each of the TLR stimuli, higher levels of CCL4 mRNA were produced in ATF3^{-/-} BMDM ϕ than in ATF3-expressing BMDM ϕ (Fig. 4 and data not shown). Both LTA and poly I:C treatments yielded amounts of CCL4 mRNA that were two-fold higher in ATF3^{-/-} macrophages compared to ATF3^{+/+} cells, whereas flagellin- and LPS-stimulation produced smaller differences. Thus, ATF3^{-/-} BMDM ϕ behaved like ATF3^{-/-} peritoneal macrophages with respect to exhibiting elevated CCL4 mRNA levels compared to corresponding ATF3^{+/+} macrophages.

Higher levels of CCL4 mRNA in ATF3^{-/-} BMDM ϕ were accompanied by increased expression of CCL4 protein compared to ATF3^{+/+} cells. Intracellular staining of CCL4 protein in untreated ATF3^{-/-} and ATF3^{+/+} BMDM ϕ followed by flow cytometry revealed 3-fold more CCL4-

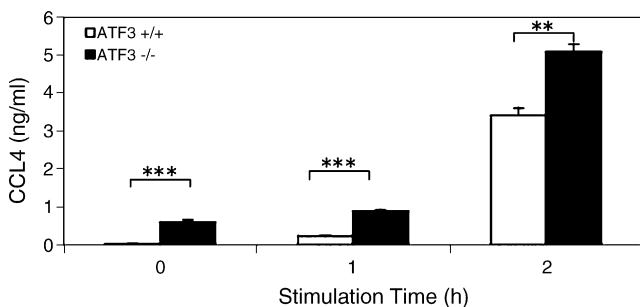


Fig. 3. Peritoneal macrophages from ATF3^{-/-} mice secrete more CCL4 than macrophages from ATF3^{+/+} mice. Thioglycollate-elicited macrophages from ATF3^{-/-} or ATF3^{+/+} mice were exposed to 10 ng/ml of LPS for the indicated time. The amount of CCL4 secreted into the culture medium was quantitated by ELISA. The data presented are the mean \pm S.E.M. from four independent samples. *t*-Test *p*-values <0.001 or <0.0001 are indicated by ** or ***, respectively.

positive ATF3^{-/-} cells than ATF3^{+/+} cells ($19.7 \pm 1.2\%$ versus $6.0 \pm 0.4\%$, mean \pm S.E.M., *N* = 4). Consequently, ATF3^{-/-} cells had a higher median fluorescence intensity than ATF3^{+/+} cells (12.20 ± 1.25 versus 7.24 ± 0.26 , mean \pm S.E.M., *N* = 4) (Fig. 5A and B). Consistent with these results, significantly more CCL4 was secreted into the culture medium by untreated ATF3^{-/-} BMDM ϕ than by ATF3^{+/+} BMDM ϕ as detected by ELISA (27.3 ± 1.2 ng/ml versus 7.2 ± 0.4 ng/ml, mean \pm S.E.M., *N* = 4) (Fig. 6). ATF3^{-/-} BMDM ϕ also produced higher induced levels of CCL4 protein than ATF3^{+/+} BMDM ϕ following stimulation with LPS (Fig. 5B), LTA or flagellin (Fig. 6). Thus, the absence of ATF3 in BMDM ϕ led

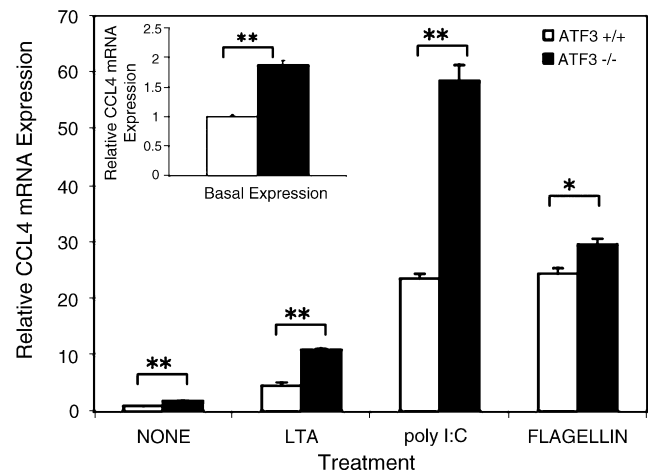


Fig. 4. Bone marrow-derived macrophages from ATF3^{-/-} mice express higher levels of CCL4 mRNA than ATF3^{+/+} BMDM ϕ . Bone marrow cells from ATF3^{-/-} and ATF3^{+/+} mice were cultured 7–10 days in CSF-enriched medium to promote macrophage differentiation. The resulting macrophage cultures were exposed to LTA (10 μ g/ml), poly I:C (25 μ g/ml) or flagellin (100 ng/ml) for 1.5 h. The relative CCL4 mRNA expression was determined as described in the legend to Fig. 2. Shown are the mean \pm S.E.M. CCL4 expression determined from quadruplicate independent samples. *t*-Test *p*-values <0.05 or <0.0001 are indicated by * or **, respectively. Similar data were obtained in a replicate experiment using a separate group of mice.

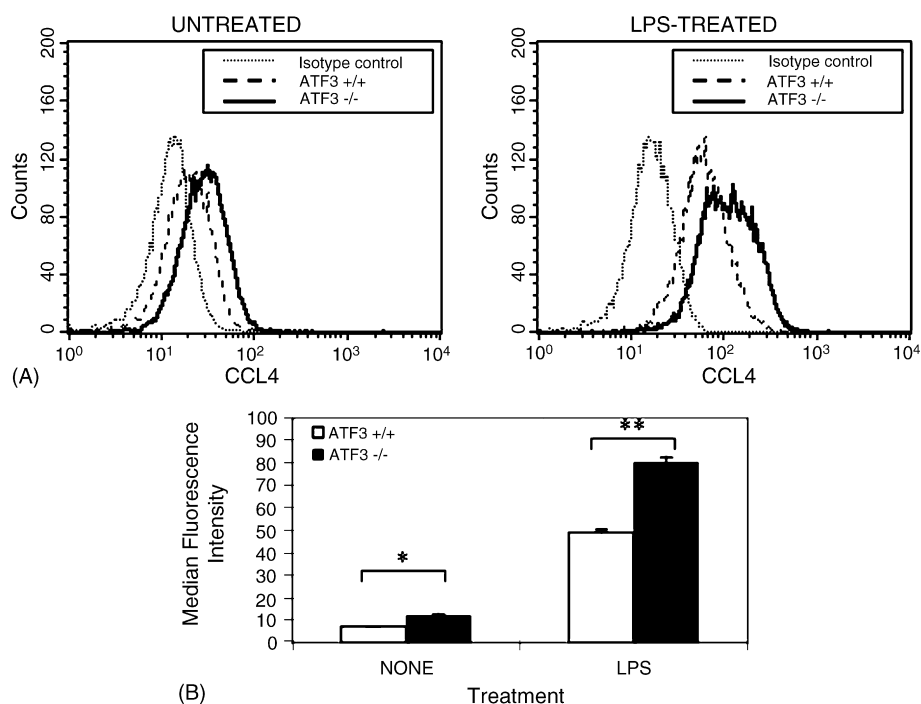


Fig. 5. CCL4 protein expression is higher in bone marrow-derived macrophages from ATF3^{-/-} mice than from ATF3^{+/+} mice. Bone marrow-derived macrophages from ATF3^{-/-} and ATF3^{+/+} mice were exposed to 100 ng/ml of LPS for 6 h in the presence of GolgiPlug reagent for the last 4 h to inhibit protein secretion. CCL4 expression was analyzed by intracellular staining with anti-CCL4 antibodies or isotype control antibodies followed by flow cytometry. (A) Representative histograms of CCL4 fluorescence from untreated and LPS-treated ATF3^{+/+} and ATF3^{-/-} macrophages are shown. (B) The median fluorescence intensity defined as the median fluorescence of anti-CCL4 antibody-stained cells minus the median fluorescence of isotype control antibody-stained cells. Results from four independent experiments were averaged and the mean \pm S.E.M. are shown. *t*-Test *p*-values <0.001 or <0.0001 are indicated by * or **, respectively.

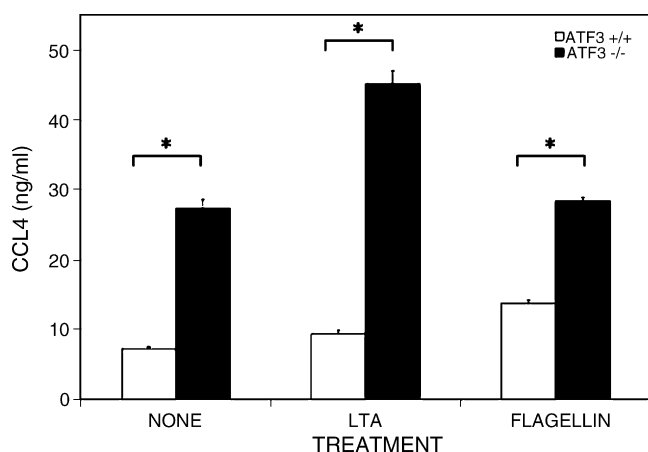


Fig. 6. Bone marrow-derived macrophages from ATF3^{-/-} mice secrete more CCL4 than macrophages from ATF3^{+/+} mice. Bone marrow-derived macrophages from ATF3^{-/-} and ATF3^{+/+} mice were exposed to LTA (10 μ g/ml) or flagellin (100 ng/ml) for 1.5 h. An ELISA was used to quantitate the amount of CCL4 secreted into the culture medium. The mean \pm S.E.M. from four experiments are shown. *t*-Test *p*-values <0.0001 are indicated by *.

to increased production of CCL4 protein in a manner similar to that observed for peritoneal M ϕ .

4. Discussion

Previous analysis of the CCL4 promoter identified an ATF/CRE sequence conserved in the human and mouse genes

that is critical for transcriptional regulation (Barabitskaja et al., 2006; Proffitt et al., 1995). At the time that the present investigation was initiated, c-Jun was the only transcription factor known to bind to this site (Proffitt et al., 1995) and the influence of c-Jun on CCL4 transcription had not been established. Consequently, further investigation of ATF/CRE site-binding proteins that regulate CCL4 transcription in macrophages was warranted.

Our ChIP and EMSA data indicate that ATF3 binds to the murine CCL4 promoter *in vivo* and that this binding is localized to the ATF/CRE site. Notably, results from both assays suggest that ATF3 is bound to the CCL4 promoter in both unstimulated and LPS-stimulated macrophages. Whether cell stimulation modulates the composition of ATF3-containing complexes requires further experiments. As an example, ATF3 and c-Jun are known to form heterodimers (Hai and Curran, 1991). It would be interesting to find out whether these two proteins bind to the CCL4 promoter as a heterodimer or as part of separate complexes in unstimulated and stimulated macrophages.

The observation that the amount of CCL4 mRNA is elevated in ATF3-deficient murine macrophages relative to congenic ATF3^{+/+} controls suggests that ATF3 functions as a transcriptional repressor of the CCL4 gene in these cells. The repressive effect of ATF3 on the CCL4 gene is consistent with the reported effect of this protein on the transcription of several other genes (Hai et al., 1999), including the ELAM-1 gene that contains an identical ATF/CRE sequence within its promoter (Kaszubska et al., 1993; Nawa et al., 2000). Our data indicate that ATF3 dampens both basal CCL4 and induced CCL4 gene expression

during the early stages of cell stimulation following exposure to LPS or other TLR stimuli. However, the influence of ATF3 on CCL4 gene expression appears to diminish as the length of cell stimulation increases beyond a few hours. Presumably, other regulatory proteins become more prominent in governing CCL4 expression at these later stimulation times. The inducible cAMP early repressor ICER that was recently reported to inducibly bind to the CCL4 ATF/CRE site and to suppress transcription of this gene in phytohemagglutinin-stimulated human T cells (Barabitskaja et al., 2006) is a potential candidate for playing this role in macrophages.

CCL4 is known to be present in the tissues directly affected by several chronic inflammatory diseases including Alzheimer's disease, Type 1 diabetes, osteoarthritis, uveitis, Kawasaki disease and chronic bronchitis (Menten et al., 2002). Published studies using neutralizing antibodies against CCL4 in some inflammatory disease models have established that this chemokine contributes to the host tissue damage associated with these pathological states (Bless et al., 2000; Manczak et al., 2002). These observations underscore the importance of understanding how CCL4 expression is controlled within the body and suggest that therapeutic manipulation of CCL4 expression could potentially be clinically useful. The results presented in this report suggest that ATF3 functions to suppress the amount of CCL4 secreted by macrophages, possibly to reduce the risk of damaging inflammatory responses.

During the preparation of this report, Gilchrist et al. (2006) published that ATF3 negatively regulates IL-6 and IL-12 gene expression in macrophages. They further showed that ATF3^{-/-} mice are more sensitive to LPS-induced septic shock than congenic ATF3-expressing mice. These observations are consistent with our findings presented in this report. Taken together, ATF3 has been demonstrated to repress the expression of several pro-inflammatory cytokine genes, suggesting that ATF3 functions as a modulator of inflammation.

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References

Barabitskaja, O., Foulke, J.S.J., Pati, S., Bodor, J., Reitz, M.S.J., 2006. Suppression of MIP-1beta transcription in human T cells is regulated by inducible cAMP early repressor (ICER). *J. Leukoc. Biol.* 79, 378–387.

Bless, N.M., Huber-Lang, M., Guo, R.F., Warner, R.L., Schmal, H., Czernak, B.J., Shanley, T.P., Crouch, L.D., Lentsch, A.B., Sarma, V., Mulligan, M.S., Friedl, H.P., Ward, P.A., 2000. Role of CC chemokines (macrophage inflammatory protein-1 beta, monocyte chemoattractant protein-1, RANTES) in acute lung injury in rats. *J. Immunol.* 164, 2650–2659.

Broxmeyer, H.E., Sherry, B., Lu, L., Cooper, S., Oh, K.O., Tekamp-Olson, P., Kwon, B.S., Cerami, A., 1990. Enhancing and suppressing effects of

recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. *Blood* 76, 1110–1116.

Buratowski, S., Chodosh, L.A., 1996. Mobility shift DNA-binding assay using gel electrophoresis. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), *Current Protocols in Molecular Biology*. John Wiley and Sons, Hoboken, NJ, pp. 12.2.1–12.2.11.

Gallily, R., Feldman, M., 1967. The role of macrophages in the induction of antibody in X-irradiated animals. *Immunology* 12, 197–206.

Gilchrist, M., Thorsson, V., Li, B., Rust, A.G., Korb, M., Kennedy, K., Hai, T., Bolouri, H., Aderem, A., 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 441, 173–178.

Hai, T., Curran, T., 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3720–3724.

Hai, T., Hartman, M.G., 2001. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 273, 1–11.

Hai, T., Wolfgang, C.D., Marsee, D.K., Allen, A.E., Sivaprasad, U., 1999. ATF3 and stress responses. *Gene Express.* 7, 321–335.

Hartman, M., Lu, D., Kim, M., Kociba, G., Shukri, T., Buteau, J., Wang, X., Frankel, W., Guttridge, D., Prentki, M., Grey, S., Ron, D., Hai, T., 2004. Role of activating transcription factor 3 in stress-induced beta-cell apoptosis. *Mol. Cell. Biol.* 24, 5721–5732.

Hooft van Huijsduijnen, R., Whelan, J., Pescini, R., Becker-Andre, M., Schenk, A.M., DeLamarter, J.F., 1992. A T-cell enhancer cooperates with NF-kappa B to yield cytokine induction of E-selectin gene transcription in endothelial cells. *J. Biol. Chem.* 267, 22385–22391.

Kaszubska, W., Hooft van Huijsduijnen, R., Ghersa, P., DeRaemy-Schenk, A.M., Chen, B.P., Hai, T., DeLamarter, J.F., Whelan, J., 1993. Cyclic AMP-independent ATF family members interact with NF-kappa B and function in the activation of the E-selectin promoter in response to cytokines. *Mol. Cell Biol.* 13, 7180–7190.

Kitchens, R.L., Ulevitch, R.J., Munford, R.S., 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J. Exp. Med.* 176, 485–494.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_t} method. *Methods* 25, 402–408.

Manczak, M., Jiang, S., Orzechowska, B., Adamus, G., 2002. Crucial role of CCL3/MIP-1alpha in the recurrence of autoimmune anterior uveitis induced with myelin basic protein in Lewis rats. *J. Autoimmun.* 18, 259–270.

Menten, P., Wuyts, A., Van Damme, J., 2002. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 13, 455–481.

Nawa, T., Nawa, M., Cai, Y., Zhang, C., Uchimura, I., Narumi, S., Numano, F., Kitajima, S., 2000. Repression of TNF-alpha-induced E-selectin expression by PPAR activators: involvement of transcriptional repressor LRF-1/ATF3. *Biochem. Biophys. Res. Commun.* 275, 406–411.

Proffitt, J., Crabtree, G., Grove, M., Daubersies, P., Bailleul, B., Wright, E., Plumb, M., 1995. An ATF/CREB-binding site is essential for cell-specific and inducible transcription of the murine MIP-1 beta cytokine gene. *Gene* 152, 173–179.

Takeshita, S., Kaji, K., Kudo, A., 2000. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *J. Bone Miner. Res.* 15, 1477–1488.

Tebo, J., Der, S., Frevel, M., Khabar, K.S., Williams, B.R., Hamilton, T.A., 2003. Heterogeneity in control of mRNA stability by AU-rich elements. *J. Biol. Chem.* 278, 12085–12093.

Weinstein, S.L., Gold, M.R., DeFranco, A.L., 1991. Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 88, 4148–4152.

Widmer, U., Manogue, K.R., Cerami, A., Sherry, B., 1993. Genomic cloning and promoter analysis of macrophage inflammatory protein (MIP)-2, MIP-1 alpha, and MIP-1 beta, members of the chemokine superfamily of proinflammatory cytokines. *J. Immunol.* 150, 4996–5012.

- Wolpe, S.D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D.G., Nguyen, H.T., Moldawer, L.L., Nathan, C.F., Lowry, S.F., Cerami, A., 1988. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J. Exp. Med.* 167, 570–581.
- Yi, T.-L., Wilman, C.L., 1989. Cloning of the murine *c-fgr* proto-oncogene cDNA and induction of *c-fgr* expression by proliferation and activation factors in normal bone marrow-derived monocytic cells. *Oncogene* 4, 1081–1087.
- Ziegler, S.F., Tough, T.W., Franklin, T.L., Armitage, R.J., Alderson, M.R., 1991. Induction of macrophage inflammatory protein-1 beta gene expression in human monocytes by lipopolysaccharide and IL-7. *J. Immunol.* 147, 2234–2239.