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ERK Activation Following Macrophage FcγR Ligation Leads to Chromatin Modifications at the IL-10 Locus[1]

Mark Lucas, Xia Zhang, Vikram Prasanna, and David M. Mosser[2]

We have previously demonstrated that macrophages stimulated in the presence of immune complexes produce high levels of IL-10. We now examine the mechanism of IL-10 superinduction. We report that the enhanced production of IL-10 correlates with a rapid and enhanced activation of two MAPKs, ERK and p38. The inhibition of either ERK or p38 prevented IL-10 induction, indicating that both MAPKs were required for IL-10 synthesis. By chromatin immunoprecipitation assay, we demonstrate that activation of ERK leads to the phosphorylation of serine 10 on histone H3 at the *il-10* gene, making the promoter more accessible to transcription factors generated in response to p38 activation. Inhibition of ERK activation prevented histone modifications, and decreased the binding of Sp1 and STAT3 to the IL-10 promoter. We conclude that the activation of ERK following FcγR ligation leads to a remodeling of the chromatin at the *il-10* locus, making it more accessible to transcription factors. The rapid and transient regulation of transcription factor accessibility to the IL-10 promoter by MAPK activation represents a novel way that the production of this cytokine is regulated. *The Journal of Immunology,* **2005, 175: 469–477.**

Activated macrophages are major mediators of inflammation. These cells secrete a variety of inflammatory cytokines, lipid mediators, and potentially toxic radicals of oxygen and nitrogen (reviewed in Ref. 1). Consequently they can contribute to the pathology associated with a number of autoimmune diseases, such as rheumatoid arthritis (2), multiple sclerosis (3), and inflammatory bowel disease (4) to mention a few. We have recently shown that macrophages activated in the presence of immune complexes secreted high levels of IL-10. The IL-10 produced by these cells could reverse lethal endotoxemia (5) and prevent Th1-type adaptive immune responses (6). Thus, immune complexes give rise to a potent anti-inflammatory population of macrophages that produce large amounts of IL-10. In the present work, we examine the mechanism of IL-10 induction in these macrophages.

Several transcription factors have been implicated in IL-10 transcription. Sp1 has been shown to play an important role in IL-10 transcription, and an Sp1 responsive element in the IL-10 promoter was localized at −89 to −78 (7). The STAT3 transcription factor has also been shown to bind to an element in the human IL-10 promoter, and a dominant negative form of STAT3 has been shown to diminish IL-10 transcription (8). A role for C/EBP has been suggested (9), although it may not be required for IL-10 transcription (7). Despite the identification of these transcription factors, the regulation of IL-10 transcription in macrophages remains somewhat elusive. One of the reasons for ambiguity is that the common stimulus used to induce IL-10 in macrophages is LPS. This compound is not a particularly potent stimulator of IL-10 production, typically inducing only in the hundred-picogram range of IL-10, which correlates with modest increases in IL-10 transcription. Thus, assays to identify transcriptional elements within the IL-10 promoter are not particularly robust. In the present study, we take advantage of the fact that stimulating macrophages in the presence of immune complexes induces the production of high levels of IL-10. This creates a robust model in which to study IL-10 transcription. Importantly, immune complexes alone do not induce cytokine production from macrophages (5). However, when these complexes are combined with an inflammatory stimulus such as LPS, the two stimuli synergize to induce high levels of IL-10. The mechanism of this superinduction is the topic of the present study.

We have previously reported that activation of macrophages in the presence of immune complexes also results in an abrogation of IL-12 production (10). The synthesis of IL-12 and its regulation are more well understood than IL-10. There have been several reports describing the down-regulation of IL-12 subsequent to macrophage stimulation. The ligation of any number of macrophage receptors, including the FcγRs (11), complement receptors (12), scavenger receptors (11), as well as G protein-coupled receptors (13) have all been linked to suppressed IL-12 production, regardless of the stimuli. Similarly, ligation of β_2 adrenergic receptors (14) and exposure of macrophages to vitamin D_3 (15), or to macrophage stimulatory protein (16), can also inhibit IL-12 production. Finally, infection of macrophages or dendritic cells with a variety of microorganisms, including measles virus (17), herpes virus (18), *Leishmania* (19), or mycobacteria (20) can all result in a reduction of IL-12 secretion. The molecular mechanism for this inhibition may be quite complex. Regardless of the mechanism(s), virtually all of the inhibition appears to occur at the level of IL-12 transcription, resulting in decreased mRNA production and decreased or absent protein secretion. In the present studies, the down-regulation of IL-12 in response to immune complexes is used as a control, to contrast what occurs at the *il-10* gene.

Having identified the reciprocal alteration in the production of IL-10 (increase) and IL-12 (decrease) by immune complexes (6), we began to study the molecular aspects of cytokine gene expression. We reasoned that the identification of the mechanism whereby these two cytokines are regulated could lead to the development of novel therapeutic strategies that would predictably

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alter macrophage phenotypes, thereby influencing innate and adaptive immunity. In the present work, we show that FcγR ligation of macrophages leads to ERK activation. Activation of ERK leads to transient modifications in the chromatin at the IL-10 locus. These alterations to the chromatin are necessary to allow the efficient transcription of IL-10, which occurs in macrophages that are stimulated in the presence of immune complexes.

Materials and Methods

Mice

Six- to 8-wk-old BALB/c mice were purchased from Taconic Farms. All mice were maintained in HEPA-filtered Thoren units (Thoren Caging Systems) at the University of Maryland (College Park, MD). Mice were used at 6–10 wk of age as a source of bone marrow-derived macrophages (BMMϕ).[3] All procedures were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee.

Reagents

The p38 MAPK inhibitor, SB203580, and the MEK/ERK inhibitor, PD98059, were purchased from Calbiochem. The JNK inhibitor peptide I was purchased from Alexis, USA (San Diego, CA). Washed SRBC were purchased from Lampire. Rabbit IgG Ab to SRBC (anti-SRBC IgG) was purchased from Cappel. Ultra pure LPS from *Escherichia coli* K12 strain was obtained from InvivoGen. Anti-phospho-H3 and acetyl-H3 Abs and chromatin immunoprecipitation (ChIP) assay kits were purchased from Upstate Biotechnology. Anti-p38 (phospho-T180/Y182), anti-STAT3, and anti-Sp1 Abs were purchased from Abcam. Anti-p38 (total), anti-ERK1/2 (total and phospho-T202/Y204), and anti-JNK (phospho-T183/Y185) were purchased from Cell Signaling Technology.

Cells

BMMϕ were prepared as previously described (6). Briefly, bone marrow was flushed from the femurs and tibias of mice and cells were plated in petri dishes in DMEM/F12 supplemented with 10% FBS, penicillin/streptomycin, glutamine, and 10% conditioned medium from the supernatant of M-CSF secreting L929 (LCM) fibroblasts. Cells were fed on day 2, and complete medium was replaced on day 6. Cells were used at 7–10 days for experiments. The RAW264.7 macrophage cell line (American Type Culture Collection) was maintained in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin and glutamine (Invitrogen Life Technologies).

Immune complexes

IgG-opsonized erythrocytes (E-IgG) were generated by incubating SRBC with anti-SRBC IgG at nonagglutinating titers for 30 min at room temperature while rotating. Opsonized cells were washed once in HBSS (Invitrogen Life Technologies) and resuspended in complete medium. E-IgG were added to macrophages at a ratio of 10:1 E-IgG to macrophages. For some experiments IgG-OVA was used as the immune complex. IgG-OVA was prepared as previously described (6).

Cell stimulation assays

For cytokine analysis, 3×10^5 macrophages per well were plated overnight in a 48-well plate in DMEM/F12. Cells were then washed and activated with either 10 ng/ml LPS alone or in combination with a 10:1 ratio of E-IgG to macrophages. Supernatants were harvested ~20 h later. Cytokines were measured by ELISA using the following Ab pairs from BD Pharmingen: IL-12p40, C15.6 and C17.8; IL-10, JES5-2A5 and JES5-16E3; TNF-α, G281-2626 and MP6-XT22.

Generation of small interfering RNA (siRNA) and cell transfections

To generate siRNA for p38α, ERK1, and ERK2, the Silencer siRNA Construction kit was used (Ambion) following the manufacturer's guidelines. The oligonucleotide sequences used to generate siRNA templates were: p38 sense 5′-AACTGGCACTTCACGATCCTGCCTGTCTC-3′, antisense 5′-ACAGGATCGTGAAGTGCCAGCCTGTCTC-3′; ERK1 sense 5′-ACTTGATGGCCACTCTGGTCCCTGTCTC-3′, antisense 5′-AAGACCAGAGTGGCCATCAAGCCTGTCTC-3′; ERK2 sense 5′-AAAACTCGAACTTTGTTGAGACCTGTCTC-3′, antisense 5′-AATCTCAACAAAGTTCGAGTTCCTGTCTC-3′. For cell transfections, 5×10^6 primary BMMϕ were transfected on day 6 with 100 nM siRNA using the Amaxa Nucleofector system and stimulated 48 h later. Gene silencing was confirmed by TaqMan real-time PCR using the following primer sequences: p38 sense 5′-CAGGATCGTGAAGTGCCAGAA-3′, antisense 5′-GCCCTCGGAGGATCTGGTA-3′ and ERK1 sense 5′-TGTTATAGGCATCCGAGACATCCT-3′, antisense 5′-CCATGAGGTCCTGAACAATGTAAAC-3′.

Western blotting

A total of 2×10^6 BMMϕ per well were plated overnight in 60-mm culture dishes. Cells were then washed and activated with 10 ng/ml LPS alone or in combination with E-IgGs in a final volume of 1 ml DMEM/F12 without L929 condition medium. Cells were then lysed in ice-cold lysis buffer (100 mM Tris, pH 8, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 containing complete EDTA-free protease inhibitor mix from Roche Diagnostics), 1 mM sodium vanadate, 50 mM sodium fluoride, and 1 mM PMSF and left on ice for 30 min. Lysates were cleared by centrifugation (13,000 rpm, 10 min, 4°C). Equal amounts of protein were loaded onto 12% SDS-polyacrylamide gels. After separation, protein was transferred to polyvinylidene difluoride membrane for 2 h. Membranes were then blocked with 5% milk powder in TBS-Tween 20 (0.1%) for 1 h at room temperature, washed briefly and incubated with primary Abs (1/1000 in 5% BSA in TBS-Tween 20) overnight at 4°C. Membranes were washed and incubated with secondary Ab (1/5000) for 1 h at room temperature and visualized using Lumi-LightPLUS chemiluminescent substrate (Roche Diagnostics).

EMSA

Nuclear extracts were prepared from 2×10^7 macrophages using the Nuclear Extraction kit (Panomics) following the manufacturer's protocol. EMSAs were conducted using the EMSA "Gel-Shift" kit (Panomics). Briefly, 5 μg of nuclear extract were incubated with biotin-Sp1 or STAT3 probe with or without unlabeled probe for 30 min at 20°C, and then run on a 6% polyacrylamide gel. Oligonucleotide-protein complexes were transferred to Biodyne B membrane (Pall). Following transfer, membranes were incubated with streptavidin-HRP and protein visualized by chemiluminescence.

Luciferase assay

RAW264.7 macrophages (4×10^6) were transfected with 5 μg of pGL-IL10-luciferase reporter plasmid (7), which is a generous gift of Dr. S. Smale (Howard Hughes Medical Institute, University of California, Los Angeles, CA) with the Amaxa Nucleofector system. After transfection, 3×10^5 cells were plated per well in 48-well culture plates. After 24 h, cells were washed and stimulated, then lysed using Glo-Lysis buffer, and luciferase activity was measured using the Bright-Glo Luciferase system (Promega).

ChIP assay

ChIP assays were conducted using the ChIP Assay kit following the manufacturer's protocol (Upstate Biotechnology). Briefly, 1×10^6 BMMϕ were plated overnight in six-well plates. Cells were stimulated as described in figures, then fixed for 10 min at 37°C in 1% paraformaldehyde. Cells were washed on ice with ice-cold HBSS containing 1 mM PMSF, harvested and then lysed in SDS lysis buffer. DNA was sheared by ultrasonication using a High Intensity Ultrasonic Processor (Cole-Parmer) for $3 \times$ 10 s pulses at 20% amplitude. Lysates were cleared by centrifugation and diluted in ChIP dilution buffer. Lysates were precleared using salmon sperm DNA/protein A-agarose and a sample of "input DNA" was collected at this point. Protein-DNA complexes were immunoprecipitated with 5 μg of Ab overnight at 4°C. Ab-protein-DNA complexes were then captured using salmon sperm DNA/protein A-agarose for 1 h at 4°C. After washing beads with low and high salt, LiCl, and TE buffers, the protein/DNA complexes were eluted using 1% SDS, 0.1 M $NaHCO_3$ buffer and disrupted by heating at 65°C for 4 h. DNA was then extracted using phenol/chloroform extraction and ethanol precipitation. PCR was conducted using promoter specific primers: IL-10 promoter (Sp1 binding region, −294 to −73) sense 5′-CAGCTGTCTGCCTCAGGAAATACAA-3′, antisense 5′-TATTCAGGCTCCTCCTCCCTCTTCT-3′ (94°C, 15 s; 60°C, 30 s; 72°C, 1 min, 35 cycles); IL-10 promoter (STAT3 binding region, −649 to −448) sense 5′-TCATGCTGGGATCTGAGCTTCT-3′, antisense 5′-CGGAAGTCACCTTAGCACTCAGT-3′ (94°C, 15 s; 56°C, 30 s; 72°C, 1 min, 35 cycles); IL-12p40 promoter sense 5′-CAAATCTGGGAGGCAGGAAAC-3′, antisense 5′-CAAAGCAAACCTTTCTATCAAATACACA-3′ (94°C, 15 s;

[3] Abbreviations used in this paper: BMMϕ, bone marrow-derived macrophage; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; E-IgG, IgG-opsonized erythrocyte.

56°C, 30 s; 72°C, 1 min, 35 cycles). Numerical designations are according to GenBank accession number M84340. PCR products were separated on 2% agarose gels. For relative quantitation of promoter levels, real-time PCR was performed.

Real-time PCR

Real-time PCR was conducted with the ABI Prism 7700 Sequence Detection System using SYBR Green PCR reagents (Applied Biosystems) following the manufacturer's guidelines. Melting curve analyses were performed after PCR amplification to ensure that a single product with the expected melting curve characteristics was obtained. In addition to the primers used for IL-10 ChiP assays as mentioned, one additional pair of primers used for the control element located −1563 to −1427 of the *IL-10* gene was sense 5′-CAGTCAGGAGAGAGGGCAGTGA-3′ and antisense 5′-TTTCCAACAGCAGAAGC AAC-3′.

DNase I sensitivity assayed by real-time PCR

DNase I accessibility was determined as previously described (21, 22), with minor modifications. Briefly, cells grown in 100-mm tissue culture dishes were stimulated at different time intervals, and formaldehyde was added for 15 min at room temperature at a final concentration of 1%. Glycine (0.125 M) was added to neutralize formaldehyde. Cells were washed and lysed in 4 ml of ice-cold Nuclei EZ lysis buffer (Sigma-Aldrich). Cells were scrapped into conical tube, centrifuged at 500 × *g* for 4 min, and the nuclei were resuspended with an additional 4 ml of ice-cold Nuclei EZ lysis buffer. Washed nuclei were pooled and resuspended in ice-cold DNase I buffer (100 mM NaCl, 50 mM Tris, pH 8.0, 3 mM $MgCl_2$, 0.15 mM spermine, and 0.5 mM spermidine) supplemented with 1 mM $CaCl_2$. DNase I (Roche Diagnostics) was then added and incubated at 37°C for 2 min. The reaction was stopped by adding equal volume of DNase I stop buffer (containing 10 mM EDTA, 20% SDS, and 0.4 M NaCl) and incubated at 65°C for 4 h to reverse cross-links. Proteinase K (100 μg) and RNase A (10 μg) were then added at 37°C overnight. DNA was purified with phenol/chloroform extraction and ethanol precipitation. Real-time PCR was conducted as previously described (22, 23).

Results

Stimulation of macrophages in the presence of immune complexes results in the augmented activation of two MAPK, p38 and ERK

We examined the magnitude and the kinetics of MAPK activation in BMMϕ, following stimulation in the presence or absence of immune complexes. BMMϕ were stimulated with LPS alone or in combination with E-IgG, and cells were lysed at various intervals thereafter and total protein extracts were analyzed by Western blotting using phospho-specific Abs against p38, ERK, or JNK. LPS stimulation alone resulted in relatively modest levels of MAPK activation, which peaked at 20 min and began to decline by 40 min (Fig. 1). The combination of E-IgG and LPS resulted in a rapid and prolonged activation of ERK (Fig. 1, *upper panels*) and p38 (Fig. 1, *middle panels*). Both MAPKs were strongly phosphorylated within 5 min of stimulation. There was also an increase in the total amount of p38 and ERK phosphorylation, which persisted for 40 min (ERK) or longer (p38) (Fig. 1). The magnitude of JNK phosphorylation following LPS administration was not substantially increased by the addition of immune complexes (Fig. 1). In summary, compared with stimulation with LPS alone, stimulation of macrophages in the presence of immune complexes resulted in a more rapid and enhanced activation of ERK and p38, whereas LPS-induced JNK activation was not significantly increased by the addition of immune complexes.

To determine the relative role of each stimulus in inducing MAPK activation, macrophages were stimulated with either LPS or immune complexes alone. MAPK activation in single-stimulation cells was compared with cells stimulated by both LPS and immune complexes (Fig. 2). Immune complexes efficiently activated both p38 and ERK1/2 (Fig. 2*A*), and the combination of LPS plus immune complexes resulted in no additional MAPK activation. Thus, immune complexes alone are sufficient to fully activate

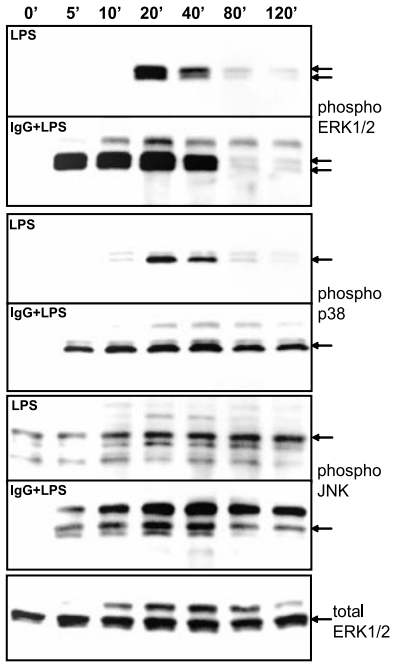

FIGURE 1. Kinetics of MAPK activation in LPS-stimulated macrophages with or without Fc receptor ligation. BMMϕ were stimulated with LPS (10 ng/ml) with or without E-IgG. Cells were then lysed at the indicated times and equal amounts of whole cell lysates were separated by SDS-PAGE and analyzed by Western blotting using phospho-specific Abs to ERK1/2, p38 or JNK. Total ERK1/2 (*bottom*) was used as the loading control. Data shown are representative of three experiments.

the MAPKs. Although immune complexes activate MAPK, they fail to induce any IL-10 production from unstimulated macrophages (Fig. 2*B*), as previously reported by us (5).

The role of MAPK activation in IL-10 induction

Having observed MAPK activation following stimulation in the presence of immune complexes, we examined the effect of inhibiting MAPK on macrophage cytokine production. We previously demonstrated that the stimulation of IFN-γ-primed macrophages in the presence of immune complexes resulted in a dramatic increase in IL-10 production (24), and a decrease in the production of IL-12 (11). We show similar data in Fig. 3, using unprimed macrophages. Stimulation of macrophages with LPS alone resulted in the production of a modest amount of IL-10, but coupling this stimulation with immune complexes caused a significant increase in IL-10 production (Fig. 3). In five separate experiments, the addition of immune complexes caused a 4.73 ± 0.45-fold increase in the production of IL-10 relative to cells stimulated with LPS alone. Stimulating macrophages with LPS alone also induced the production of the p40 subunit of IL-12 (Fig. 3, *middle panel*), and coupling this stimulation with E-IgG decreased IL-12 production to <200 pg/ml. Thus in Fig. 3, stimulating unprimed macrophages in the presence of immune complexes gave rise to a population of anti-inflammatory macrophages secreting ~10 ng/ml IL-10 and <200 pg/ml IL-12. We have previously used several different stimuli and a variety of immune complexes, both soluble and particulate, to achieve a similar reciprocal alteration in the production of these two cytokines (5, 25).

Several recent studies have demonstrated a role for MAPKs in LPS signaling for cytokine secretion (26–28). To investigate the role of MAPKs in IL-10 induction, BMMϕ were treated with specific pharmacological inhibitors of p38, ERK, or JNK before stimulation in the presence or absence of immune complexes (Fig. 3).

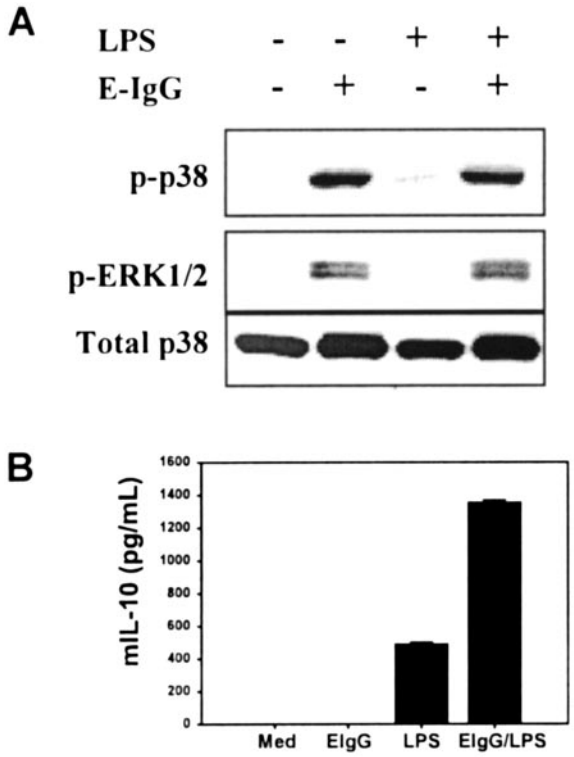

FIGURE 2. MAPK activation and IL-10 induction. *A*, BMMϕ were stimulated with LPS (10 ng/ml) with (+) or without (−) E-IgG for 15 min. Cell lysates were separated by SDS-PAGE and analyzed by Western blotting using phospho-specific Abs to p38 or ERK1/2. Total p38 (*bottom*) was used as the loading control. *B*, BMMϕ were stimulated with LPS (10 ng/ml) with or without E-IgG for 6 h. Supernatants were collected and analyzed for IL-10 production by ELISA. Data represent the mean of triplicate determinations. *, Significant difference ($p \leq 0.05$) from LPS-treated cells.

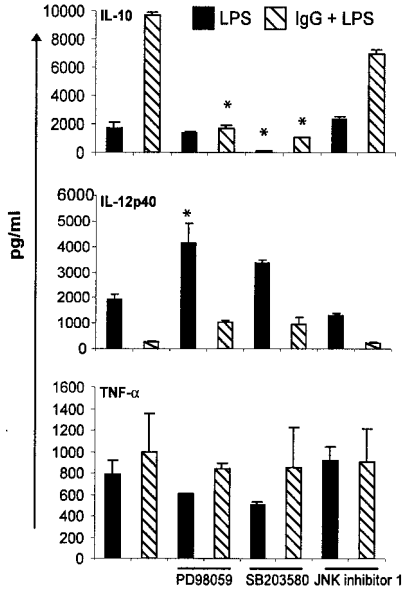

FIGURE 3. Effect of MAPK inhibition on cytokine secretion by activated macrophages. Macrophages were pretreated with PD98059 (10 μM), SB203580 (1 μM,) or JNK inhibitory peptide (1 μM) for 30 min, then stimulated with LPS (10 ng/ml) alone (■) or LPS + E-IgG (▨). Supernatants were harvested after 20 h and cytokines analyzed by ELISA. Data represent the mean + SD of triplicate samples and are representative of five individual experiments. Bars marked (*) designate inhibitor treatments that result in values that are different ($p \leq 0.05$) from control cells stimulated in the absence of inhibitors.

Inhibition of p38 with SB203580, or ERK with the MEK inhibitor PD98059, resulted in a substantial inhibition of IL-10 secretion (Fig. 3, asterisks). Inhibition of p38 prevented the LPS-induced IL-10 production, whereas inhibition of ERK appeared to prevent the superinduction of IL-10 caused by immune complexes. Neither of these inhibitors decreased IL-12 production (Fig. 3, *middle panel*). In fact, IL-12 production was actually increased by the ERK inhibitor PD98059, as previously reported (29). The decrease in IL-12 production caused by the addition of immune complexes was not affected by the inhibition of either ERK or p38. Inhibition of JNK, with the JNK inhibitor 1 peptide, had no effect on IL-10 production. This inhibitor did, however, partially inhibit IL-12p40 release. For these studies, TNF was used as a control cytokine, whose production was not reproducibly affected by any of the three MAPK inhibitors (Fig. 3, *lower panel*).

Western blotting was performed to confirm the specificity of the MAPK inhibitors. Pretreating cells with PD98059 caused a dramatic decrease in ERK1/2 activation, but did not influence p38 activation (Fig. 4*A*). Similarly, treating cells with SB203580, an inhibitor that prevents phosphorylated p38 from activating downstream targets, did not inhibit ERK1/2 activation (Fig. 4). To determine the level at which IL-10 was being inhibited, real-time PCR analysis was performed to measure IL-10 mRNA in macrophages stimulated with LPS in the presence or absence of immune complexes. Macrophages stimulated in the presence of immune complexes had an increased amount of IL-10 mRNA, relative to macrophages stimulated with LPS alone (Fig. 4*B*). This induction of IL-10 mRNA was prevented by stimulating these cells in the presence of either ERK or p38 inhibitors (Fig. 4*B*). The JNK inhibitor 1 peptide had no affect on IL-10 mRNA levels (data not

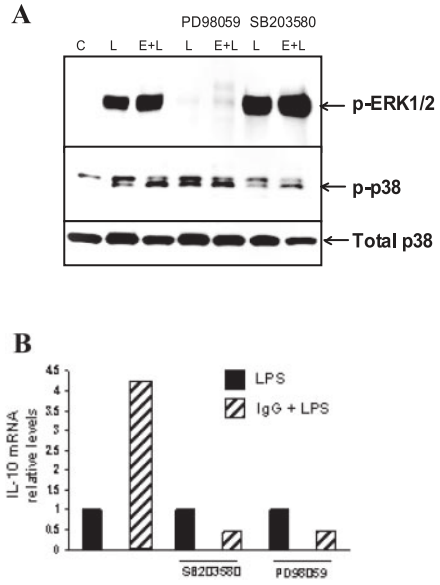

FIGURE 4. The specificity of MAPK inhibition. *A*, Western blotting of phosphorylated ERK1/2 (*upper*) and phosphorylated p38 (*middle*) following treatment with PD98059 (10 μM) or SB203580 (1 μM) is shown. Total p38 (*lower*) was used as the loading control. *B*, BMMϕ were pretreated with SB203580, PD98059, or saline for 30 min, and then stimulated with LPS (10 ng/ml) with (▨) or without (■) E-IgG for 2 h. Total RNA was reverse transcribed and analyzed by real-time PCR. Samples were normalized to a GAPDH control and normalized to stimulation with LPS alone, which value was considered to be 1.

shown). The increased IL-10 mRNA accumulation that accompanies activation in the presence of immune complexes was not due to differences in IL-10 mRNA stability (data not shown).

To confirm a specific role for the two MAPKs in IL-10 induction, siRNA specific for p38 or ERK were transfected into primary BMMϕ 48 h before stimulation. In all cases, gene silencing was confirmed by real-time PCR (data not shown). The primary isoform of p38 expressed in macrophages is p38α. siRNA to p38α siRNA almost completely abrogated IL-10 production by macrophages stimulated with either LPS alone or LPS in combination with immune complexes (Fig. 5). Macrophages express both ERK1 and ERK2. siRNA to ERK1 almost completely prevented the augmentation of IL-10 production caused by the addition of immune complexes (Fig. 5). siRNA to ERK2 decreased IL-10 levels by 50–75% (data not shown). The combination of siRNA to both ERK1 and ERK2 completely prevented the induction of IL-10 caused by immune complexes (data not shown). Thus, both ERK1 and 2 may contribute to the induction of IL-10 caused by immune complexes, but ERK1 appears to play the dominant role in this induction. MAPK p38 appears to be required for the relatively modest levels of IL-10 that are produced in response to LPS alone.

Failure of immune complexes to stimulate extrachromosomal IL-10 promoters

To determine whether the addition of immune complexes to stimulated macrophages had any effect on the binding of transcription factors to the IL-10 promoter, EMSAs were performed using Sp1 and STAT-3, which are two transcription factors that have been implicated in IL-10 synthesis (7, 8). By EMSA, stimulation of macrophages with LPS caused a significant increase in the binding of both Sp1 (Fig. 6*A*) and STAT3 (Fig. 6*B*) to their respective binding motifs in the IL-10 promoter. In both cases, this binding was specific and was efficiently competed for by a 30-fold excess of cold probe. Coupling macrophage stimulation with immune complexes did not increase the binding of either transcription factor to the IL-10 promoter. Equivalent amounts of Sp1 and STAT3 were bound to DNA following activation in the presence or absence of E-IgG. Furthermore, the inhibition of ERK activation

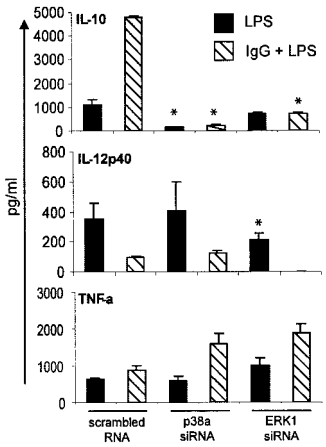

FIGURE 5. The inhibition of MAPKs by siRNA. Sequence-specific siRNA for p38α or ERK1 (100 nM) or scrambled sequence dsRNA were transfected into day 7 BMMϕ. Cells were cultured for an additional 48 h and then stimulated with LPS (10 ng/ml) alone (■) or LPS + E-IgG (▨). Supernatants were harvested after 20 h and cytokines analyzed by ELISA. Data represent the mean level of cytokine + SD of triplicate samples. *, Difference $p \leq 0.05$ relative to the scrambled RNA controls. Data are representative of three experiments.

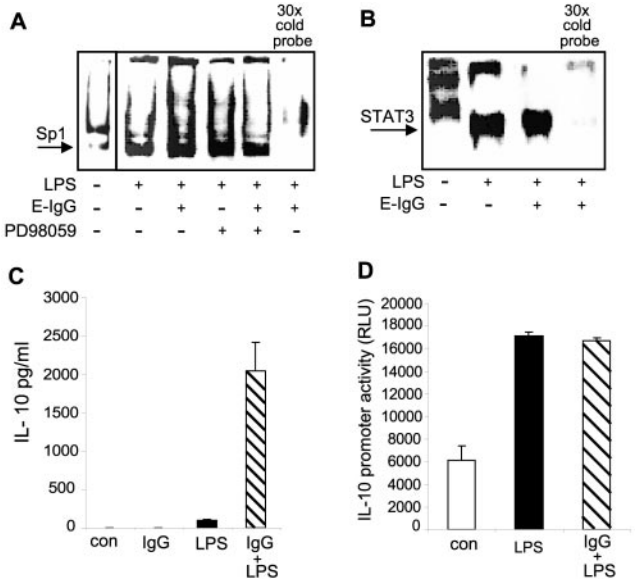

FIGURE 6. Activation of transcription factors in stimulated macrophages. *A*, Cells were stimulated with LPS (10 ng/ml) or LPS plus E-IgG for 30 min, and nuclear extracts were analyzed by EMSA using Sp1 (*A*) or STAT3 (*B*) specific probes. *C*, RAW264 cells were stimulated with medium (□), E-IgG (dotted), LPS (solid bar), or LPS+E-IgG (▨), and cytokines were measured by ELISA after 6 h. Data represent the mean of triplicate samples ± SD. *D*, RAW264 cells were transfected with pRL-IL10-luciferase reporter plasmid (5 μg/ml) and 24 h later stimulated with medium (control, □), 10 ng/ml LPS (■), or E-IgG+LPS (▨) for 4 h. Cells were then lysed and luciferase activity measured. The data presented are representative of three experiments.

with PD98059 had no effect on Sp1 binding to the IL-10 promoter (Fig. 6*A*).

Additional studies to examine IL-10 transcription were performed on RAW 264 macrophage-like cells, transfected with an IL-10 promoter luciferase reporter construct. By ELISA, RAW264 cells behaved similarly to primary macrophages, producing no IL-10 in response to immune complexes alone and only modest amounts when stimulated with LPS alone (Fig. 6*C*). Like macrophages, these cells produced much higher amounts of IL-10 when stimulated with a combination of LPS and immune complexes (Fig. 6*C*). The production of luciferase driven by an IL-10 promoter, however, did not reflect this superinduction. Unstimulated RAW cells expressed modest levels of luciferase activity (Fig. 6*D*). Stimulation of these cells with LPS resulted in a significant increase in luciferase activity (Fig. 6*D*), however this activity was not further increased by stimulation in the presence of immune complexes (Fig. 6*D*). This lack of response to immune complexes is in contrast to our previous observations with IL-12 luciferase reporter constructs, which were dramatically diminished by the addition of immune complexes (30). Thus, although immune complexes cause a dramatic increase in IL-10 secretion by macrophages, these increases were not detected by assays dependent on the regulation of extrachromosomal DNA. Neither the EMSA nor the luciferase reporter assay reflected an increase in IL-10 production following the addition of immune complexes.

Activation of MAPKs by FcγR results in histone modifications at the IL-10 promoter

To determine the mechanism whereby ERK activation leads to increased IL-10 transcription, we examined histone modifications at the IL-10 locus by ChIP assays. Histone modifications, such as phosphorylation and acetylation, are thought to be important

events in the regulation of gene expression (28). ERK in particular has been postulated to phosphorylate core histone proteins, including histone H3 (31). ChIP assays were performed on macrophages activated by LPS in the presence or absence of E-IgG. Macrophages treated with E-IgG had higher levels of phosphorylated H3 (serine 10) associated with the IL-10 promoter relative to resting (control) or LPS stimulated macrophages (Fig. 7*A*). Similar to ERK activation shown in Fig. 2, immune complexes alone were sufficient to increase IL-10 promoter-associated histone phosphorylation, and the addition of LPS to immune complexes increased this phosphorylation only slightly. We also performed ChIP assays using an Ab specific to acetylated lysines on histone H3. A similar pattern of increased acetylated histone H3 associated with the IL-10 promoter was observed following FcγR ligation, although the amount of acetylation was substantially more modest (Fig. 7*B*). Both phosphorylation and acetylation were time-dependent events. Phosphorylation occurred rapidly and peaked at 30 min, whereas the more modest levels of histone H3 acetylation persisted for 1–2 h poststimulation (Fig. 7*B*). To correlate histone phosphorylation with ERK activation, similar studies were performed on macrophages that were stimulated with a soluble immune complex, IgG-OVA, in the presence or absence of PD98059 to prevent ERK activation. Similar to E-IgG, the addition of IgG-OVA caused a dramatic increase in histone phosphorylation, and this increase was completely abrogated by inhibiting ERK1/2 activation with PD98059 (Fig. 7*C*). ERK inhibition reduced the amount of histone phosphorylation at this locus to background levels (Fig. 7*C*). The inhibition of p38 with SB203580 had a more modest effect on histone H3 phosphorylation, decreasing it substantially, but not reducing it to background levels (Fig. 7*C*).

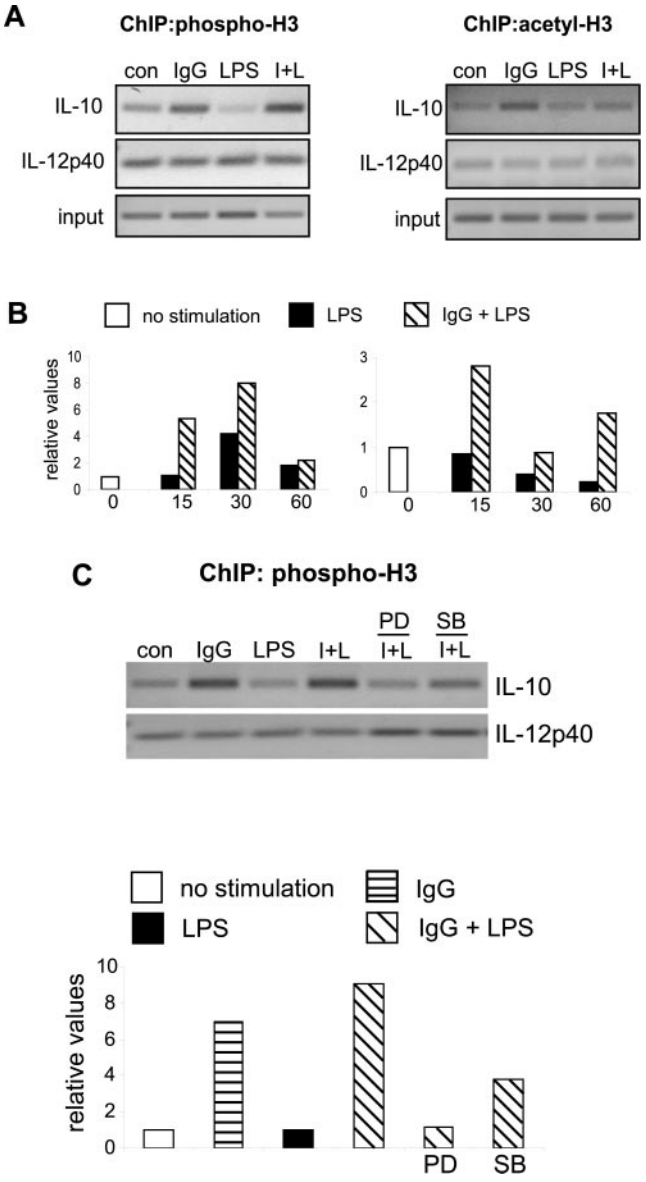

FIGURE 7. ChIP analysis of histone H3 modifications at the *il-10* promoter in stimulated macrophages. BMMϕ were stimulated with medium, LPS, E-IgG, or E-IgG + LPS for 30 min (*A*) or the indicated times (*B*). ChIP assays were conducted as described in *Materials and Methods*. Immunoprecipitations were performed using Abs specific to phosphorylated H3 (IP phospho-H3) (*left*) or IP acetyl-H3 (*right*), and conventional RT-PCR (*A*) or quantitative real-time PCR (*B*) was performed using primers specific to the *il-10* or *il-12* promoters. For real-time PCR, samples were normalized to input DNA controls (*lower lane*). *C*, Cells were pretreated with PD98059 (10 μM), SB203580 (1 μM), or medium for 30 min and then stimulated with medium (□), IgG-OVA (dotted bar), LPS (■), or both (▨) for 40 min. ChIP analysis was conducted using a phosphorylated H3-specific Ab. Conventional RT-PCR was conducted using primers specific to the *il-10* or *il-12* promoters. Real-time PCR samples were normalized to input DNA controls.

We examined the specificity of histone modifications following activation of macrophages in the presence of immune complexes. The IL-12(p40) promoter was used as a control. Histones associated with the IL-12 promoter were neither phosphorylated nor acetylated in response to immune complexes (Fig. 7, *A* and *C*). Therefore FcγR signaling results in histone modifications that are specific to the IL-10 promoter. To determine the fine specificity of nucleosome modifications, the 12 successive nucleosomes located 5′ of the IL-10 transcriptional start site were individually analyzed for modifications following stimulation. Data from three of these sites are shown in Fig. 8. The two nucleosomes comprising the Sp1 (Fig. 8*A*) and the STAT3 (Fig. 8*B*) sites underwent rapid and extensive increases in histone H3 phosphorylation following the addition of immune complexes. This phosphorylation was transient and reduced to baseline levels within 1 h poststimulation, a time at which acetylation peaks (Fig. 8). A similar analysis was performed on a control nucleosome located ~1500 bp away from the transcriptional start site. There was little detectable increase in either phosphorylation or acetylation at this site (Fig. 8*C*). The lack of histone modifications at this site was comparable to that which occurred at the IL-12 promoter (data not shown).

Transcription factor binding to the IL-10 promoter in situ

ChIP assays were performed to examine the binding of Sp1 and STAT3 to the IL-10 promoter in situ within live cells (Fig. 9*A*). Control (unstimulated) cells exhibited virtually no binding of either Sp1 or STAT3 to the IL-10 promoter. Similarly, the addition of immune complexes to resting cells, a condition that does not induce IL-10 production from macrophages (see Fig. 2*B*), also failed to result in transcription factor binding to the IL-10 promoter (Fig. 9*A*). Stimulation of cells with LPS alone, a condition that induces low levels of IL-10 production, caused a modest increase in Sp1 binding to the IL-10 promoter, but no detectible STAT3 binding. However, the addition of LPS plus immune complexes induced the efficient binding of both Sp1 and STAT3 to the IL-10 promoter. Thus, the ChIP assays for Sp1 and STAT3 binding to the IL-10 promoter were accurate reflections of IL-10 transcription. Both transcription factors bound to the IL-10 promoter in situ under conditions of IL-10 superinduction. Furthermore, inhibiting ERK activation with PD98059, a condition that prevented IL-10 induction, reversed transcription factor binding to the IL-10 promoter to background levels (Fig. 9*B*).

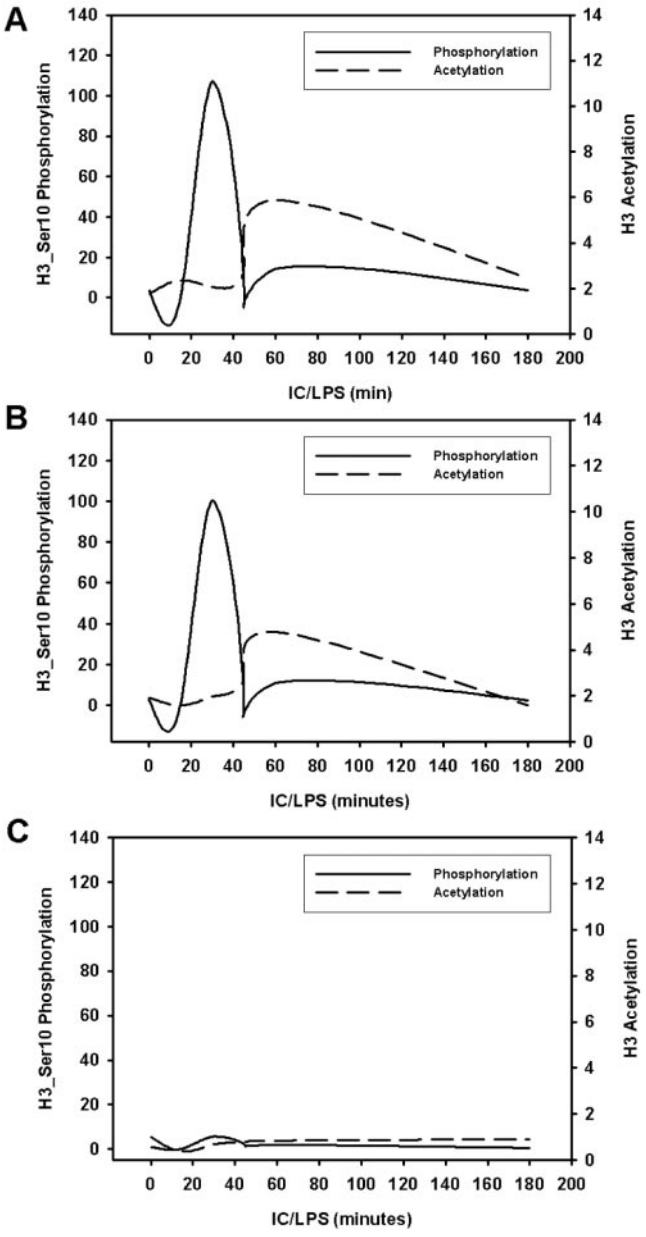

FIGURE 8. Dynamic changes in histone modifications at the *il-10* promoter region. Quantitative real-time PCR analysis of three regions of murine IL-10 promoter was performed following ChIP assays using Abs to either acetylated or phosphorylated histone H3. The fold change in each case is presented as a comparison of the experimental sample at each time relative to a corresponding control sample precipitated with normal rabbit serum. H3 serine 10 phosphorylation is designated by the *left* y-axis. H3 acetylation is designated by the *right* y-axis. Each sample was quantitated in duplicate on two separate occasions. *A*, Histone H3 association with the Sp1 binding site of the modified IL-10 promoter region located between −294 and −73. *B*, Histone H3 association with the STAT3 binding site of IL-10 proximal promoter region located between −704 and −603. *C*, Histone H3 association with IL-10 5′-flanking region located between −1563 and −1427.

To correlate transcription factor binding with DNA accessibility, chromosomal DNA in resting and activated cells was digested with DNase I. In resting cells, the IL-10 promoter was relatively resistant to DNase I treatment (Fig. 10). In cells exposed to immune complexes, either alone or in combination with LPS, however, there was a rapid increase in the sensitivity to DNase I treatment. This sensitivity was induced within 30 min of stimulation,

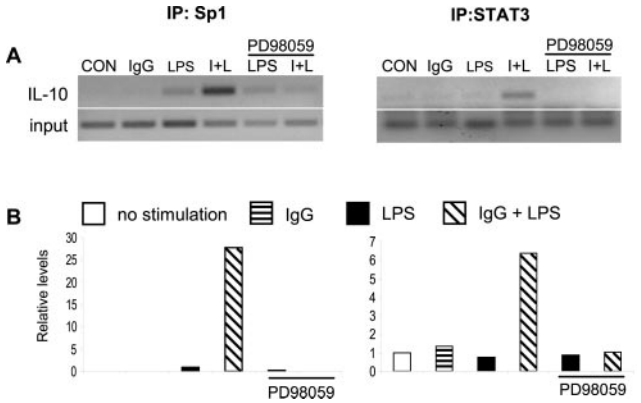

FIGURE 9. ChIP analysis of Sp1 and STAT3 binding at the IL-10 promoter in stimulated macrophages. Macrophages were stimulated with medium (□), E-IgG (▤), LPS (■), or LPS + E-IgG (▧) for 40 min. Some cells were pretreated with PD98059 (10 μM). Immunoprecipitations were conducted using either Sp1 (IP:Sp1) or STAT3 (IP:STAT3) specific Abs. *A*, Conventional RT-PCR was conducted using primers that amplify the putative Sp1 or STAT3 binding sites of the IL-10 promoter, respectively. *B*, Real-time PCR analysis of samples obtained in *A*. Samples were normalized to input DNA controls.

and it persisted for the entire 3 h observation period. Thus, immune complexes alone are sufficient to activate ERK, to modify chromatin, and to make the *il-10* locus more accessible. These immune complexes, however, are not sufficient to induce IL-10 expression. The induction of IL-10 requires a second signal, which is provided by stimulating the cells to activate the transcription factors that bind to the IL-10 promoter.

Discussion

We have previously shown that activation of macrophages in the presence of immune complexes increases the production of IL-10 and reduces IL-12 production (10). This gives rise to a population of macrophages with potent anti-inflammatory properties. We have termed these cells type II activated macrophages. We have previously shown that this response to immune complexes occurs in macrophages taken from a variety of different species, including mice and human, from various anatomic locations, including the peritoneum, lung, and blood (25). The response also occurs following many different types of macrophage stimulation, including LPS, lipoteichoic acid, and CD40L (5), and in the presence of several different soluble or particulate immune complexes (6, 10). Thus, we feel that this response to FcγR ligation is a universal response that is a general property of most, if not all, macrophages. In the present work we sought to determine the mechanism whereby IL-10 was induced in response to immune complexes.

Immune complexes alone were not sufficient to induce IL-10. Rather cytokine production required both the stimulation (LPS) and the addition of immune complexes. Only the combination of these two stimuli resulted in high levels of IL-10 production. Therefore, we examined signal transduction in macrophages, following the addition of each stimulus alone or in combination. LPS alone signals through TLRs to induce NF-κB translocation and moderate levels of MAPK activation, as previously described (32). Although these signals were sufficient to maximally activate extrachromosomal IL-10 constructs, LPS alone induced only modest levels of IL-10 secretion by macrophages. This low level of IL-10 could be completely blocked by inhibiting p38 (Fig. 3). The coupling of LPS with immune complexes, however, resulted in a substantial increase in IL-10 production. Thus, signals generated via FcγRs converge with those generated by TLRs to induce high

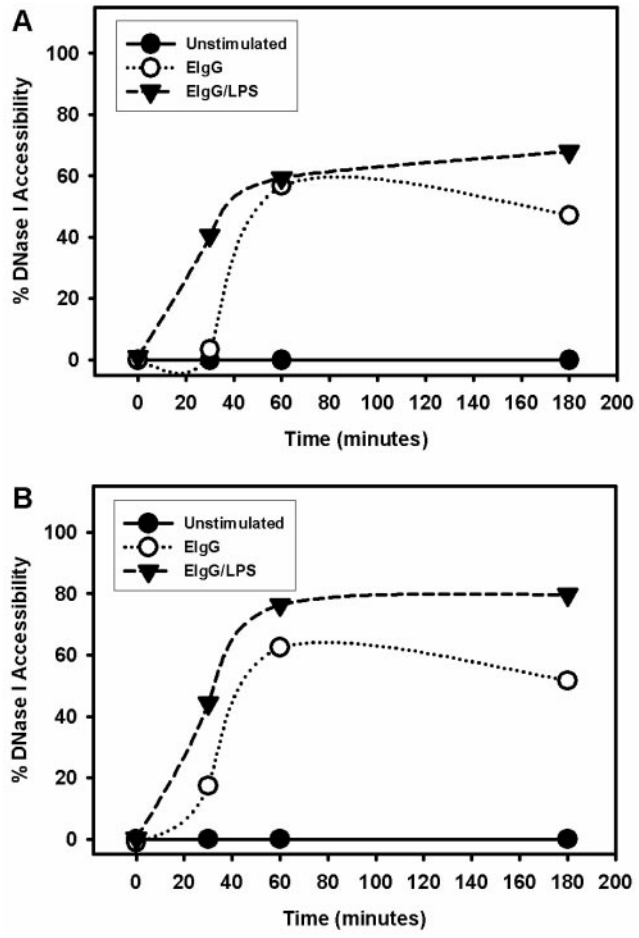

FIGURE 10. Changes in DNase I accessibility at the modified IL-10 promoter region. Quantitative real-time PCR analysis was performed on two regions of the IL-10 promoter following brief exposure of nuclei to DNase I. Macrophages were stimulated with E-IgG alone (○) or in combination with LPS (▼) and compared with unstimulated cells (●). Cells were analyzed at various times poststimulation and expressed as a percentage of accessibility, as described in *Materials and Methods*. *A*, Changes of DNase I accessibility of the Sp1 binding region modified IL-10 promoter located between −294 and −73. *B*, Changes of DNase I accessibility of the STAT3 binding region of the IL-10 promoter located between −704 and −603.

levels of IL-10. We show that FcγR ligation caused a rapid increase in ERK activation. This activation was required for IL-10 production, but not sufficient. ERK activation had to be coupled with an inflammatory stimulus to induce IL-10. The inflammatory stimuli activate the myriad transcription factors that drive cytokine and costimulatory molecule expression. In the absence of ERK activation, however, these activated transcription factors fail to effectively induce IL-10 production. In the present work, we show that activation of ERK makes the *il-10* promoter accessible to these transcription factors, resulting in the production of high levels of IL-10.

Several groups have correlated cytokine production with MAPK activation (28) and some investigators have recently suggested that differential activation of the MAPKs may lead to differences in cytokine production (33). We confirm the observation of Mathur et al. (33) that p38 activation is linked to inflammatory cytokine production, and that ERK activation can lead to the production of IL-10. In the present work we show that the mechanism of IL-10 induction is the activation of ERK, which leads to chromatin modifications at the *il-10* locus, to make the promoter more accessible to transcription factors that bind to it.

It has been well established that covalent modifications to chromatin, including acetylation, phosphorylation, methylation, and even ubiquitination can influence gene expression (34). In fact, some have suggested that the specific combination of histone modifications represents a code that can determine gene expression (35). In the case of differentiated lymphocytes, some of these modifications can lead to long term heritable changes in gene expression, which can define the very phenotype of the cell (36). Epigenetic changes in gene expression have been associated with T cell deviation along the Th1 or Th2 pathway. In fact, a recent study has demonstrated that IL-10 chromatin becomes altered as T cells commit to the Th2 lineage (37). The alterations described in the present work also depend on chromatin alterations, and appear to use some of the same types of histone modifications that lead replicating cells to undergo these epigenetic changes in gene expression. In the present situation, however, these changes occur quite rapidly, and their effect is transient. Alterations to chromatin are observed within the first 15 min of stimulation, and they can be reversed as quickly as 1 h later (Fig. 8). Furthermore, in end-stage cells such as macrophages, these need not be heritable alterations that can be passed on to daughter cells, and therefore their effect is transient and reversible.

Although histone phosphorylation was originally associated with chromatin condensation and gene silencing during cell division (38), several studies have correlated histone modifications and specifically phosphorylation of the serine 10 residue on histone H3 with transcriptional activation (28, 39). In fact, a human genetic disease, Coffin-Lowry syndrome, is characterized by impaired transcription of c-*fos* and defective histone H3 phosphorylation (40). The acetylation of histones has also been linked to transcriptional activation (41), and frequently histone acetylation occurs in association with histone phosphorylation. In yeast phosphorylation often precedes and can be a prerequisite for histone acetylation (42), whereas in *Drosophila* these two modifications may be independently regulated (43). Although the dramatic increase in early histone H3 phosphorylation, following exposure of macrophages to immune complexes, requires ERK activation, it is unlikely that ERK directly modifies chromatin. Rather, several histone H3 kinases have been identified that represent candidates for the observed phosphorylation events. Research is underway to identify the IL-10-associated histone kinase. Importantly, the alterations to chromatin that we observe appear to be restricted to the *il-10* gene, in that no such modifications are observed at the *il-12* gene. Further analyses to determine the mechanism of this modification are underway.

The increase in IL-10 production following activation in the presence of immune complexes makes these macrophages potent anti-inflammatory cells (5). In the present work we show that stimulation of cells with LPS alone leads to modest levels of ERK activation, modest binding of Sp1 to the IL-10 promoter in situ, and only low levels of IL-10 gene expression. Coupling stimulation with FcγR ligation, however, leads to increased ERK activation, histone H3 modifications at the *il-10* locus, and dramatic increases in Sp1 and STAT3 binding to the IL-10 promoter. We conclude that these modifications are required for the high levels of IL-10 that are produced by macrophages activated in the presence of immune complexes, and suggest that manipulating MAPK activation in macrophages can change the phenotype of the activated macrophage.

Disclosures

The authors have no financial conflict of interest.

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