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Gene-Specific Repression of Proinflammatory Cytokines in Stimulated Human Macrophages by Nuclear IκBα

Chandra C. Ghosh,*†1 Sitharam Ramaswami,*†1 Ashish Jucekar,* Hai-Yen Vu,* Luciano Galdieri,* Dennis Davidson,‡ and Ivana Vancurova*†

We have previously shown that increased nuclear accumulation of IκBα inhibits NF-κB activity and induces apoptosis in human leukocytes. In this study, we wanted to explore the possibility that the nucleocytoplasmic distribution of IκBα can be used as a therapeutic target for the regulation of NF-κB–dependent cytokine synthesis. Treatment of LPS-stimulated human U937 macrophages with an inhibitor of chromosome region maintenance 1-dependent nuclear export, leptomycin B, resulted in the increased nuclear accumulation of IκBα and inhibition of NF-κB DNA binding activity, caused by the nuclear IκBα-p65 NF-κB interaction. Surprisingly, however, whereas mRNA expression and cellular release of TNF-α, the β form of pro-IL-1 (IL-1β), and IL-6 were inhibited by the leptomycin B–induced nuclear IκBα, IL-8 mRNA expression and cellular release were not significantly affected. Analysis of in vivo recruitment of p65 NF-κB to NF-κB–regulated promoters by chromatin immunoprecipitation in U937 cells and human PBMCs indicated that although the p65 recruitment to TNF-α, IL-1β, and IL-6 promoters was inhibited by the nuclear IκBα, p65 recruitment to IL-8 promoter was not repressed. Chromatin immunoprecipitation analyses using IκBα and SS36 phosphospecific p65 NF-κB Abs demonstrated that although the newly synthesized IκBα induced by postinduction repression is recruited to TNF-α, IL-1β, and IL-6 promoters but not to the IL-8 promoter, SS36-phosphorylated p65 is recruited to IL-8 promoter, but not to TNF-α, IL-1β, or IL-6 promoters. Together, these data indicate that the inhibition of NF-κB–dependent transcription by nuclear IκBα in LPS-stimulated macrophages is gene specific and depends on the SS36 phosphorylation status of the recruited p65 NF-κB. The Journal of Immunology, 2010, 185: 000–000.

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Address correspondence and reprint requests to Dr. Ivana Vancurova, Department of Biological Sciences, St. John’s University, 8000 Utopia Parkway, St. Albert’s Hall, Room 225, Queens, NY 11439. E-mail address: vancuroi@stjohns.edu

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; CRM1, chromosome region maintenance 1; IL-1β, β form of pro-IL-1; LMB, leptomycin B; mut, mutant; NES, nuclear export sequence; PGN, peptidoglycan; wt, wild-type.

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affected. Analysis of in vivo recruitment of p65 NF-κB to the corresponding promoters in U937 macrophages revealed that although the p65 recruitment to TNF-α, IL-1β, and IL-6 promoters is inhibited by the nuclear IκBα, recruitment to IL-8 promoter is not repressed. A similar mechanism is operating also in primary human PBMCs, because the p65 NF-κB recruitment to TNF-α promoter is inhibited by the LMB-induced nuclear IκBα, whereas the p65 recruitment to IL-8 promoter is not repressed. Interestingly, the IL-8 promoter is specifically associated with the S536-phosphorylated form of p65 NF-κB, whereas only the unphosphorylated p65 is recruited to promoters of TNF-α, IL-1β, and IL-6 genes. These findings indicate that the S536 phosphorylation of p65 NF-κB regulates the ability of nuclear IκBα to inhibit the NF-κB binding to promoters and provide novel insights into the mechanisms by which the nuclear IκBα regulates the NF-κB–dependent transcription in human leucocytes.

Materials and Methods

Abs and reagents

Purified polyclonal Abs against human IκBα (sc-371), NF-κB–p65 (sc-372), phosphorylated NF-κB–p65 on S536 (sc-33020), NF-κB–p50 (sc-7178), and recombinant human IκBα protein (sc-4094) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Actin Ab was from Sigma-Aldrich (St. Louis, MO), HRP-conjugated anti-rabbit and anti-mouse secondary Abs were from Santa Cruz Biotechnology.

T4 polynucleotide kinase, polydeoxyinosinic-deoxyctydilic acid, and Sephadex G25 spin columns were purchased from Pharmacia (Piscataway, NJ). CREB (sc-2504, sc-2517), AP-1 (sc-2501, sc-2514), and NF-κB (sc-2505, sc-2511) gel shift oligonucleotides were from Santa Cruz Biotechnology. [32P]-ATP was purchased from PerkinElmer (Waltham, MA). Peptidoglycan (PGN), LPS, LMB, and all other reagents were molecular biology grade and purchased from Sigma-Aldrich.

Cell culture

U937 human promyeloid cells were obtained from American Type Culture Collection (Rockville, MD). The cells were grown in RPMI 1640 medium (Invitrogen, Green Island, NY) supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM l-glutamine, and antibiotics at 37°C in a 5% CO2 humidified atmosphere as described previously (28, 29). The study was approved by the Human Subjects Review Committee of the North Shore-Long Island Jewish Health Care System (New Hyde Park, NY), and informed written consent was obtained from all the subjects.

EMSA

EMSA assays were performed in nuclear extracts as described (28–31). For competition or supershift experiments, binding reactions were performed in the presence of 30 M excess unlabeled oligonucleotide or 1 μg specific polyclonal Ab or rabbit Ab to NF-κB protein. The resulting complexes were resolved on 4% nondenaturing polyacrylamide gels that had been prerun at 150 V for 1 h in 0.5 × TBE buffer. Electrophoresis was conducted at 150 V for 3 h. Postelectrophoresis, gels were transferred to Whatman DE-81 paper, dried, and analyzed by a PhosphorImager.

Western blot analysis

Nuclear extracts were prepared as described previously (28). Denatured proteins were separated on 12% denaturing polyacrylamide gels, and immunoblotting analysis was performed as described (28–31). The images were analyzed by densitometry by using image analysis software (UNSCAN-IT gel version 5.1, Silk Scientific, Orem, UT) as described (28, 29).

Real-time PCR

Total RNA was isolated by using the RNeasy mini-kit (Qiagen, Valencia, CA) as described (32). The iScript one-step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) was used as a supermix, and 20 ng RNA was used as template on a Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). The primers used for quantification of TNF-α, IL-1β, IL-6, IL-8, and IL-10 mRNA were purchased from SA Biosciences (Frederick, MD).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analyses were performed by using the protocol from Upstate Biotechnology (Millipore, Billerica, MA) with minor modifications (33). Briefly, cellular proteins and DNA were cross-linked by adding formaldehyde to the growth medium to a final concentration of 1% for 10 min at 37°C, and glycine was added at a final concentration of 0.125 M to neutralize formaldehyde. Cells were washed with PBS containing protease inhibitors (1 mM PMSF, aprotinin [1 μg/ml], and pepstatin A [1 μg/ml]), scraped, and collected by centrifugation. Cells were then resuspended in SDS lysis buffer, incubated at 4°C for 10 min, and sonicated. The lysates were centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatants were diluted 10-fold with ChIP dilution buffer and precleared with salmon sperm DNA/protein A agarose (Upstate Biotechnology, Lake Placid, NY) for 30 min at 4°C. Immunoprecipitation was performed overnight at 4°C, with Abs against p65 NF-κB, p50 NF-κB, or IκBα. Following immunoprecipitation, the samples were incubated with salmon sperm DNA/protein A agarose for 1 h, and the immune complexes were collected by centrifugation (1000 rpm at 4°C), washed, and extracted with 1% SDS/0.1 M NaHCO3. The cross-linking was reversed by heating with 5 M NaCl at 65°C for 4 h. Chromatin-associated proteins were digested with proteinase K, and the samples were extracted with phenol/chloroform, followed by precipitation with ethanol. The pellets were resuspended in nuclease-free water and subjected to real-time PCR. One eighth of the immunoprecipitated DNA was analyzed by real-time PCR (25 μl reaction mixture) using the iQ SYBR Green Supermix and the Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated by using the ChIP–qPCR Human IGX1A Negative Control Assay (SA Biosciences) as a negative control and corrected for the efficiency of the primers, which detect specific genomic DNA sequences within open reading frame-free intergenic regions, or promoter deserts, lacking any known or predicted structural genes. The primers used for real-time PCR were the following: TNF-α forward, 5′-GCTTCCTCCAGATGACCT-3′; reverse, 5′-TCTGTCCTATTAGACCA-3′; IL-1β forward, 5′-CGCCATAAGCATGGTTTCTT-3′; reverse, 5′-GCAGGATGAGCTTTGTTTTGCTT-3′; IL-10: forward, 5′-TGGGAGTCAATGCAGAGTGGC-3′; reverse, 5′-GGCCAGTTCTTTGCTTCCAGTGGC-3′; and IL-6: forward, 5′-TAGAGGAGTCTTTCCTTCCCGTG-3′; reverse, 5′-TGCTCTTCTCCCGTG-3′.

Nuclear coimmunoprecipitation

Nuclear extracts were prepared using the Active Motif’s Nuclear Complex Co-IP Kit (54001) as described previously (30). The nuclear extracts were incubated (4°C, overnight) with p65 NF-κB Ab (sc-372) to capture rabbit preimmune IgG (sc-2027) as described previously (30). The immune complexes were immunoprecipitated on A/G Plus Agarose (sc-2034) with the protocol from Upstate Biotechnology (Millipore, Billerica, MA), washed four times with PBS buffer, resolved on 10% SDS gel, and examined by Western blot analysis.

ELISA

The cytokine release was measured in cell culture supernatants by commercially available ELISA kits (R&D Systems, Minneapolis, MN) as previously described (32).

Statistical analysis

The results represent at least three independent experiments. Numerical results are presented as means ± SE. Data were analyzed by using an InStat software package (GraphPad, San Diego, CA). Statistical significance was evaluated by using Mann-Whitney U test with Bonferroni correction for multiple comparisons, and p < 0.05 was considered significant, denoted by an asterisk.

Results

LMB inhibition of cytokine release from stimulated U937 cells is cytokine specific

To determine whether LMB inhibits the cellular release of proinflammatory cytokines from stimulated human U937 monocytic cells, U937 cells were differentiated with PMA for 24 h and stimulated with LPS (1 μg/ml) or PGN (10 μg/ml) in the presence or absence of 10 nM LMB for indicated times (0–6 h). As previously reported (34, 35), both LPS and PGN induced a release of TNF-α, IL-1β, IL-6, and IL-8 from PMA-differentiated U937 macrophages. However, whereas appreciable amounts of TNF-α and IL-8 were...
released within 2 h poststimulation, IL-1β and IL-6 were released only 6 h poststimulation and in concentrations 10–100 times lower than TNF-α and IL-8 (Fig. 1).

LMB significantly reduced the release of TNF-α, IL-1β, and IL-6 from both LPS- and PGN-stimulated U937 cells (Fig. 1A–C). In contrast, LMB did not have any substantial effect on the release of IL-8 from LPS- or PGN-stimulated cells (Fig. 1D). Fig. 2 illustrates the dose dependence of TNF-α and IL-8 release from differentiated U937 cells stimulated 6 h with LPS on LMB. Whereas the release of TNF-α was significantly inhibited by 10 nM LMB (Fig. 2A), the release of IL-8 was not substantially affected (Fig. 2B).

**LMB inhibits TNF-α, IL-1β, and IL-6, but not IL-8, mRNA synthesis**

We next analyzed whether LMB regulates the cytokine release at the level of transcription. U937 cells were differentiated with PMA and stimulated with LPS in the absence or presence of 10 nM LMB, and mRNA levels were measured by real-time PCR. As shown in Fig. 3, whereas the maximal levels of TNF-α and IL-8 mRNA were achieved within 2 h after LPS stimulation, synthesis of IL-1β and IL-6 was induced at later time points and continued increasing 6 h poststimulation. However, whereas the maximal mRNA levels of TNF-α and IL-8 in LPS-stimulated cells were ~20 times higher compared with unstimulated cells, mRNA levels of IL-1β and IL-6 6 h poststimulation were ~40 and 80 times higher, respectively, compared with unstimulated cells (Fig. 3).

During LPS stimulation, mRNA levels of TNF-α, IL-1β, and IL-6 were significantly inhibited by LMB, whereas IL-8 mRNA was not reduced (Fig. 3), and thus, the mRNA data were in good agreement with the cytokine release results (Fig. 1). This LMB inhibition of cytokine expression was not restricted to proinflammatory cytokines, because the mRNA levels of anti-inflammatory cytokine IL-10 were inhibited by LMB as well (data not shown). These results indicated that LMB inhibits cytokine release by inhibiting transcription; however, this inhibition of cytokine transcription is gene specific.

**FIGURE 1.** Regulation of proinflammatory cytokine release from stimulated U937 macrophages by LMB. U937 cells (5 × 10⁶) were differentiated with 10 ng/ml PMA for 24 h and stimulated with LPS (1 µg/ml) or PGN (10 µg/ml) in the absence or presence of 10 nM LMB. The cytokine release was measured in cell culture supernatants by ELISA. A, Release of TNF-α. B, Release of IL-1β. C, Release of IL-6. D, Release of IL-8. Data represent the mean (± SE) of four independent experiments, and the statistical analysis was made in comparison with LPS or PGN alone. *p < 0.05.

**LMB inhibits NF-κB activity by inducing nuclear accumulation of IκBα, which then binds to p65 NF-κB in the nucleus**

Transcriptional regulation of TNF-α, IL-1β, IL-6, and IL-8 involves the transcription factors NF-κB, CREB, and AP-1 (36–39). Thus, we sought to determine whether LMB regulates the NF-κB, CREB, and AP-1 DNA-binding activities in LPS-stimulated U937 macrophages. Differentiated U937 cells were stimulated with LPS in the presence or absence of 10 nM LMB for up to 6 h. As illustrated in Fig. 4A and 4B, 10 nM LMB inhibited the inducible NF-κB DNA-binding activity, whereas it did not have any effect on the constitutive DNA-binding activities of CREB or AP-1. Using supershift assays, we determined that this inducible NF-κB DNA-binding activity consisted of NF-κB p65/50 heterodimer, whereas the lower DNA binding band represented p50/50 NF-κB homodimer (Fig. 4C). rIκBα protein bound to the p65/50 heterodimer, but not to the p50/50 homodimer (Fig. 4C, top panel). The competition assays shown in the bottom panels of Fig. 4C confirmed the DNA binding specificity of AP-1 and CREB complexes. Together, these results indicated that the LMB inhibition of cytokine transcription involves the transcription factor NF-κB, but not AP-1 or CREB.

To investigate the mechanisms by which LMB inhibits the NF-κB DNA-binding activity, we analyzed the nuclear protein levels of IκBα and NF-κB p50 and p65 subunits in differentiated U937 cells stimulated with LPS for up to 6 h, in the presence and absence of 10 nM LMB. As shown in Fig. 5A and 5B, in U937 cells stimulated with LPS for up to 6 h, LMB significantly increased the nuclear accumulation of the newly synthesized IκBα induced by postinduction repression, similar to what we previously observed in LPS-stimulated human neutrophils (29). LMB treatment did not inhibit the LPS-induced nuclear translocation of p50 and p65 NF-κB subunits; appreciable amounts of NF-κB p65 and p50 proteins were found in the nucleus of LMB-treated cells (Fig. 5A), which exhibited a significant decrease in NF-κB DNA-binding activity (Fig. 4A). These results indicated that LMB inhibits NF-κB activity by inducing the nuclear accumulation of IκBα, which then binds to NF-κB and inhibits its transcriptional activity.
in comparison with LPS alone (LMB, 0 nM).

FIGURE 2. Dose response of TNF-α and IL-8 release from LPS-stimulated U937 macrophages on LMB. U937 cells (5 × 10⁵) were differentiated with 10 ng/ml PMA for 24 h, stimulated 6 h with LPS (1 μg/ml) in the presence of increasing concentrations of LMB, and the TNF-α (A) and IL-8 (B) release was measured by ELISA. Data represent the mean (± SE) of four independent experiments. The statistical analysis was made in comparison with LPS alone (LMB, 0 nM). *p < 0.05.

To determine whether the LMB-induced nuclear IκBα binds to p65 NF-κB in the nucleus, we performed a coimmunoprecipitation experiment by using p65 NF-κB Ab and nuclear extracts prepared from U937 cells stimulated 6 h with LPS in the presence or absence of 10 nM LMB. As illustrated in Fig. 5C, top panel, p65 was immunoprecipitated from the nuclear extracts of LPS-stimulated cells by using p65 NF-κB Ab but not by control preimmune IgG. No p65 was found in the immunoprecipitates from the nuclear extracts of unstimulated cells, corresponding to the absence of p65 in the nucleus in unstimulated cells and confirming specificity of the p65 Ab. Immunoblotting using IκBα Ab (Fig. 5C, bottom panel) showed that IκBα was coimmunoprecipitated with p65 from the nuclear extracts of LPS-stimulated cells treated with LMB, whereas less IκBα was recovered from the nuclear extract of LPS-treated cells in the absence of LMB, corresponding to the lower nuclear levels of IκBα in cells stimulated with LPS without LMB (Fig. 5A). No IκBα was found in the extracts immunoprecipitated with control IgG (Fig. 5C, bottom panel). Together, these data show that IκBα that accumulates in the nucleus after LMB treatment associates with the nuclear p65 NF-κB and provide the mechanism by which the nuclear IκBα inhibits NF-κB DNA-binding activity.

Inhibition of p65 recruitment to NF-κB–regulated genes by LMB-induced nuclear IκBα is gene specific

Our results demonstrating that LMB inhibits NF-κB DNA-binding activity (Fig. 4) and mRNA synthesis of TNF-α, IL-1β, and IL-6, but not IL-8 (Fig. 3) suggested that the inhibition of NF-κB–dependent transcription by nuclear IκBα is gene specific. To test this possibility, we analyzed the p65 NF-κB in vivo recruitment to TNF-α, IL-1β, IL-6, and IL-8 promoter regions by ChIP. Differentiated U937 macrophages were stimulated with LPS in the
immunoprecipitation with IκBα, as well as NF-κB p50 and p65 Abs, in unstimulated as well as LPS-stimulated (6 h) cells in the absence and presence of 10 nM LMB.

As shown in Fig. 7, in cells that were not stimulated with LPS, neither p65 nor p50 NF-κB were recruited to TNF-α, IL-1β, IL-6, or IL-8 promoters; this is consistent with the Western data demonstrating that p65 NF-κB is not present in the nucleus of unstimulated U937 cells (Fig. 5). However, whereas the p65 NF-κB recruitment to these promoters in 6-h stimulated cells displayed 6–15-fold enrichment, depending on the type of the promoter, p50 NF-κB enrichment was only 1–4-fold. This is in agreement with studies demonstrating that p50 associates with promoter regions mainly through p56 NF-κB (5, 6). Interestingly, the lowest p50 recruitment in the case of TNF-α and IL-8 promoters (Fig. 7A, 7D) was associated with the lower mRNA levels (Fig. 3), suggesting that mainly p65/66 NF-κB homodimers are recruited in vivo to TNF-α and IL-8 promoters in LPS-stimulated U937 macrophages, and this is associated with a lower level of transcriptional induction. The p50 NF-κB recruitment to IL-1β and IL-6 promoters was reduced by LMB, similar to the recruitment of p65 NF-κB (Fig. 7B, 7C). Together, these data indicate that in vivo, p50/65 NF-κB heterodimers are recruited to IL-1β and IL-6 promoters, and this binding is inhibited in the presence of IκBα.

Gene-specific recruitment of the newly synthesized IκBα to NF-κB–regulated cytokine promoters in LPS-stimulated macrophages

As expected, ChIP analysis using IκBα immunoprecipitating Ab revealed that IκBα was not recruited to either promoter in unstimulated cells (Fig. 7). Cell stimulation with LPS resulted in a 2–5-fold increase in the recruitment of the newly synthesized IκBα to TNF-α, IL-1β, and IL-6 promoters compared with unstimulated cells (Fig. 7A–C). This is consistent with the Western data showing the presence of the newly synthesized IκBα induced by post-induction repression in the nucleus of U937 cells stimulated 6 h with LPS (Fig. 5) and with studies demonstrating that IκBα associates with DNA through p65 NF-κB (5, 6). Importantly, LMB treatment that increases the nuclear accumulation of IκBα (Fig. 5A, 5B) inhibited the LPS-induced IκBα recruitment to TNF-α, IL-1β, and IL-6 promoters (Fig. 7A–C), indicating that the LMB-induced nuclear IκBα inhibits transcription of these genes by removing p65 NF-κB from the NF-κB promoter sites.

In contrast to TNF-α, IL-1β, or IL-6, enrichment of IκBα at the IL-8 promoter was not markedly affected by LPS stimulation or by LMB treatment (Fig. 7D). These results indicate that the nuclear IκBα does not bind to p65 NF-κB bound in vivo to the IL-8 promoter site. Together, these data suggest that the nuclear IκBα is able to remove transcriptionally active p65 NF-κB from TNF-α, IL-1β, and IL-6 promoters, whereas it does not inhibit p65 binding to IL-8 promoter in LPS-stimulated macrophages.

Gene-specific regulation of p65 NF-κB recruitment to TNF-α and IL-8 promoters by nuclear IκBα in primary human PBMCs

To determine whether a similar mechanism is operational in primary human cells, and to rule out possible biochemical changes initiated during the PMA-induced differentiation of U937 cells, we analyzed p65 recruitment to TNF-α and IL-8 promoters in primary human PBMCs stimulated with LPS for 4 h in the presence or absence of 10 nM LMB. As shown in Fig. 8, similar to U937 macrophages, in LPS-stimulated PBMCs, the LMB-induced nuclear IκBα significantly inhibited p65 NF-κB recruitment to the TNF-α promoter, but not to the IL-8 promoter. This is in good agreement with our previous observation that LMB inhibits TNF-α but not IL-8 release from LPS-stimulated PBMCs (32).
and demonstrates that the gene-specific inhibition of p65 NF-κB recruitment by nuclear IκBa is not limited to U937 cells, but a similar mechanism is operational in primary human PBMCs as well.

**FIGURE 6.** Effect of LMB on p65 NF-κB recruitment to TNF-α, IL-1β, IL-6, and IL-8 promoters in LPS-stimulated U937 macrophages. U937 cells (5 × 10⁶) were differentiated with 10 ng/ml PMA for 24 h and stimulated with LPS (1 μg/ml) in the absence or presence of 10 nM LMB for 0–6 h. The in vivo p65 recruitment to NF-κB–dependent promoters was measured by ChIP analysis and quantified by real-time PCR. A, p65 recruitment to TNF-α promoter site. B, p65 recruitment to IL-1β promoter. C, p65 recruitment to IL-6 promoter. D, p65 recruitment to IL-8 promoter. The data are presented as the change in occupancy over the human IGX1A (SA Biosciences) sequence control and represent the mean (± SE) of four independent experiments. The statistical analysis was made in comparison with LPS alone. *p < 0.05.

**FIGURE 7.** Recruitment of IκBa and NF-κB p50 and p65 proteins to the promoter regions of TNF-α, IL-1β, IL-6, and IL-8 genes. U937 cells (5 × 10⁶) were differentiated with 10 ng/ml PMA for 24 h and stimulated with LPS (1 μg/ml) in the absence or presence of 10 nM LMB for 0 and 6 h. The in vivo IκBa, p50, and p65 recruitment to TNF-α (A), IL-1β (B), IL-6 (C), and IL-8 (D) promoters was measured by ChIP analysis and quantified by real-time PCR. The data are presented as the change in occupancy over the human IGX1A (SA Biosciences) sequence control and represent the mean (± SE) of four independent experiments. The statistical analysis was made in comparison with LPS alone. *p < 0.05.

**FIGURE 8.** Effect of LMB on p65 NF-κB recruitment to TNF-α and IL-8 promoters in LPS-stimulated primary human PBMCs. PBMCs (2.5 × 10⁶) were stimulated with LPS (100 ng/ml) in the absence or presence of 10 nM LMB for 4 h. The in vivo p65 recruitment to TNF-α, IL-1β, or IL-6 promoters was measured by ChIP analysis and quantified by real-time PCR. The data are presented as the change in occupancy over the human IGX1A (SA Biosciences) sequence control and represent the mean (± SE) of four independent experiments. The statistical analysis was made in comparison with LPS alone. *p < 0.05.

S536-phosphorylated p65 NF-κB is specifically recruited to IL-8, but not to TNF-α, IL-1β, or IL-6 promoters in LPS-stimulated U937 macrophages. To uncover the mechanism responsible for the differential inhibition of p65 NF-κB recruitment to NF-κB–dependent promoters by nuclear IκBa, we analyzed recruitment of p65 NF-κB phosphorylated on S536, because this phosphorylation was previously described to result in a decreased affinity for IκBα binding in vitro (40–43). Differentiated U937 cells were stimulated with LPS for 2 and 6 h, and the recruitment of S536 p65 NF-κB was analyzed by ChIP. Whereas the S536 p65 was not recruited to TNF-α and IL-1β, or IL-6 promoters in 2- (Fig. 9A) and 6-h (Fig. 9B) stimulated macrophages, it was recruited to the IL-8 promoter during both times (Fig. 9). Together, these data indicate that TNF-α and IL-8 are both regulated by p65/65 homodimers. However, whereas the p65
NF-κB occupying TNF-α promoter is unphosphorylated and can be removed by IκBα, the p65 recruited to IL-8 promoter is phosphorylated on S536, which decreases its affinity for IκBα and makes it unresponsive to the inhibition by nuclear IκBα (Table I).

Discussion

The major finding of this study is that the nuclear IκBα has a differential effect on the in vivo expression of NF-κB-regulated proinflammatory genes. Whereas transcription of TNF-α, IL-1β, and IL-6 in LPS-stimulated human macrophages is inhibited by the nuclear IκBα, transcription of IL-8 is not. We have previously shown that the increased nuclear accumulation of IκBα inhibits in vitro NF-κB DNA binding and expression of NF-κB-dependent antiapoptotic genes (29–31). In this study, we show that LMB inhibits proinflammatory cytokine release from stimulated human macrophages (Fig. 1) by inducing nuclear accumulation of IκBα (Fig. 5A, 5B), which then associates with p65 NF-κB in the nucleus (Fig. 5C), resulting in the inhibition of NF-κB DNA-binding activity (Fig. 4) and inhibition of transcription (Fig. 3). However, quantitative ChIP analysis of the in vivo p65 NF-κB recruitment to NF-κB–dependent promoters revealed that the regulation of NF-κB–dependent transcription by LMB-induced nuclear IκBα is gene specific. Whereas the LMB-induced nuclear IκBα inhibits p65 NF-κB recruitment to TNF-α, IL-1β, and IL-6 promoters (Fig. 6A–C), thus inhibiting transcription of these genes (Fig. 3A–C), it does not remove p65 NF-κB from IL-8 promoter (Fig. 6D) and does not inhibit IL-8 transcription (Fig. 3D).

Importantly, our results indicate that an identical or a similar mechanism is operational in primary human leukocytes, because the LMB-induced nuclear IκBα inhibited p65 NF-κB recruitment to the TNF-α but not the IL-8 promoter in LPS-stimulated human PBMCs (Fig. 8). In addition, ChIP analysis using Abs specific against the S536-phosphorylated form of p65 NF-κB demonstrated that the S536 p65 is specifically recruited to IL-8 promoter, but not to TNF-α, IL-1β, or IL-6 promoters (Fig. 9), indicating that this phosphorylation may represent one of the mechanisms responsible for the gene-specific inhibition of NF-κB–dependent transcription by nuclear IκBα.

The regulation of NF-κB recruitment to target genes is extremely complex and depends on the cell stimulus and on the cell type that is mediating the inflammatory response (10, 44–46). For example, in LPS-stimulated murine macrophages, p65 NF-κB is recruited to the IL-6 promoter 2 h poststimulation, whereas in murine fibroblasts, it takes only 15 min (47, 48). Using quantitative ChIP analysis of the in vivo binding of NF-κB and IκBα proteins to TNF-α, IL-1β, IL-6, and IL-8 promoters, we demonstrate in this paper that both p65 and p50 NF-κB are recruited to IL-1β and IL-6 promoters in 6-h LPS-stimulated human macrophages, whereas TNF-α and IL-8 promoters contain predominantly p65 NF-κB (Fig. 7). These data indicate that the in vivo transcription of IL-1β and IL-6 genes in LPS-stimulated macrophages is regulated by p50/65 NF-κB heterodimers, whereas the synthesis of TNF-α and IL-8 is controlled by p65 homodimers. Interestingly, the TNF-α and IL-6 genes that recruited predominantly p65 NF-κB (Fig. 7A, 7D) are induced earlier (Fig. 3A, 3D) than the IL-1β and IL-6 genes (Fig. 3B, 3C) that recruited both p65 and p50 NF-κB (Fig. 7B, 7C). In addition, the IL-1β and IL-6 genes are induced to a considerably higher level than the TNF-α and IL-8 genes (Fig. 3), indicating that the p50/65 heterodimer is associated with a higher level of transcription. In macrophages stimulated 6 h with LPS, the newly synthesized IκBα induced by postinduction repression was recruited to TNF-α, IL-1β, and IL-6 promoters, but not to the IL-8 promoter (Fig. 7). These results suggest that the IL-8 promoter is not regulated by IκBα in vivo and that the regulation of NF-κB–dependent transcription by nuclear IκBα is promoter specific. This is further supported by the fact that the LMB-induced nuclear IκBα does not inhibit IL-8 transcription, whereas it inhibits transcription of TNF-α, IL-1β, and IL-6 genes (Fig. 3). Consistent with this finding, it has been previously shown in vitro that the IL-8 promoter binds p65 NF-κB homodimers, whereas it does not bind p50/65 heterodimers (49). In addition, a recent study using ChIP analysis demonstrated a selective recruitment of p65 NF-κB to the IL-8 promoter in dendritic cells (47). It is interesting to note, however, that in Jurkat cells, the portion of p65 that is specifically targeted to the IL-8 promoter is phosphorylated on S536, resulting in its inability to bind IκBα in vitro (40).

Our results indicate that the S536 phosphorylation of p65 also regulates its interaction with IκBα in vivo in LPS-stimulated macrophages, and this phosphorylation may represent one of the

Table 1. NF-κB sequences in EMSA and cytokine promoters, composition of the bound NF-κB dimers, and regulation by the nuclear IκBα

<table>
<thead>
<tr>
<th>Gene</th>
<th>NF-κB Site</th>
<th>NF-κB Proteins</th>
<th>Inhibition by IκBα</th>
</tr>
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<tr>
<td>EMSA consensus sequence</td>
<td>GGGACTTCCC</td>
<td>p50/65</td>
<td>+</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GGGCATGCCC</td>
<td>p50/65</td>
<td>+</td>
</tr>
<tr>
<td>IL-6</td>
<td>GGGATTTCCT</td>
<td>p50/65</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GGATATCCTT</td>
<td>p65/65</td>
<td>+</td>
</tr>
<tr>
<td>IL-8</td>
<td>GGAATTCCTT</td>
<td>S536-p65/S536-p65</td>
<td>–</td>
</tr>
</tbody>
</table>

+, inhibited by IκBα; −, not inhibited by IκBα.
mechanisms responsible for the gene-specific inhibition of NF-κB–dependent transcription by nuclear IκBα. The unphosphorylated p65 that associates with TNF-α, IL-1β, and IL-6 promoters is removed by binding to nuclear IκBα, whereas the S536-phosphorylated portion of p65 binds, as a homodimer, to the IL-8 promoter, independently of the nuclear IκBα. This S536 phosphorylation of p65 NF-κB has been reported to be mediated by the enzymes of IκB kinase complex and has been shown to regulate p65 acetylation and transcription activity (50–52). It might occur before p65 binds to DNA, either in the cytoplasm or in the nucleus, or after, as a part of the preinitiation complex assembly.

In addition, the strength of the nuclear IκBα-p65 NF-κB interaction might be influenced by the DNA sequence of κB response elements in the regulatory regions of NF-κB–dependent genes. In this model, the IL-8 promoter sequence may allow a stronger binding of the S536-phosphorylated p65 NF-κB, which has a significantly lower affinity for the nuclear IκBα. This would be consistent with a recent study demonstrating that a single nucleotide can influence the recruitment of specific NF-κB dimers and the required cofactors for efficient gene transcription (44, 53).

In this context, we have compared the NF-κB promoter sequences of IL-1β, IL-6, TNF-α, and IL-8 genes used in this study (Table I). Interestingly, the TNF-α and IL-8 NF-κB binding sites that recruited predominantly p65 NF-κB had C and T at the 8th and 10th position, respectively; however the IL-8 site differs from the TNF-α promoter sequence in the third position, having A instead of G (Table I). Thus, according to this model, A in the third position would increase affinity for the S536-phosphorylated p65 binding or recruitment of the corresponding kinase, which phosphorylates p65 on S536, thus decreasing its affinity for the nuclear IκBα. Future studies should determine whether p65 is phosphorylated on S536 before its binding to the IL-8 promoter or whether the corresponding kinase is preferentially recruited to IL-8 promoter, where it phosphorylates p65, thus inhibiting its interaction with IκBα.

LMB belongs to a group of CRM1 inhibitors that have been investigated for their ability to increase the nuclear accumulation of IκBα, p53, and the oncoprotein BCR-ABL in cells of myeloid origin, suggesting their anticancer and anti-inflammatory potential, earliest events in the LMB-induced changes is probably the in-

Disclosures

The authors have no financial conflicts of interest.

References


