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The p38-MSK1 Signaling Cascade Influences Cytokine Production through CREB and C/EBP Factors in Human Neutrophils

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Neutrophils influence innate and adaptive immunity by generating numerous cytokines and chemokines whose regulation largely depends on transcriptional activators such as NF-κB and C/EBP factors. In this study, we describe the critical involvement of CREB transcription factors (CREB1 and activating transcription factor-1) in this functional response as well as relevant upstream signaling components. Neutrophil stimulation with LPS or TNF led to the phosphorylation, DNA binding activity, and chemokine promoter association of CREB1 and activating transcription factor-1. These responses occurred downstream of the p38-MSK1 signaling axis, as did the phosphorylation and promoter association of another bZIP factor, C/EBPβ. Conversely, inhibition of RSK1 failed to alter the phosphorylation of either CREB1 or C/EBPβ in neutrophils. From a more functional standpoint, the inhibition of p38 MAPK or MSK1 interfered with cytokine generation in neutrophils. Likewise, overexpression of a dominant-negative CREB1 mutant (K-CREB) or of a point mutant (S133A) resulted in a decreased ability of human neutrophil-like PLB-985 cells to generate inflammatory cytokines (CXCL8, CCL3, CCL4, and TNF-α). Collectively, our data show the involvement of CREB1 in neutrophil cytokine production, the key role of its S133 residue, important upstream signaling events, and the parallel activation of another bZIP factor. These are all potential molecular targets that could be exploited in the context of several chronic inflammatory diseases that prominently feature neutrophils and their products. The Journal of Immunology, 2013, 191: 000–000.

Neutrophils play a crucial role in host immunity. Their phagocytic and microbicidal activities contribute greatly to pathogen clearance, and their ability to generate a wide array of cytokines and chemokines influences the development and evolution of both the innate and adaptive immune responses (1–3). Cytokine and chemokine production is largely dependent on transcriptional activation in neutrophils (4). In this regard, we have previously shown that NF-κB and C/EBP transcription factors are required for the induction of multiple cytokine genes in these cells (5–7), whereas AP-1 factors do not seem to play any significant role (6, 8, 9). In the latter instance, we have reported that CREB1 is the main constituent of AP-1 DNA-binding activities in human neutrophils (8), but the functional consequences of this finding have remained unclear.

The CREB family encompasses three members, termed CREB1, cAMP response element modulator (CREM), and activating transcription factor-1 (ATF-1). The latter has a high level of homology with CREB1, whereas the CREM gene can yield multiple splice variants—some acting as activators of CREB1 target genes and others as repressors of CREB1 activity (10). All members of the CREB family require dimerization through their bZIP domain for DNA binding. The high homology in their bZIP domain allows CREB1, CREM, and ATF-1 to form both homo- and heterodimers, although ATF-1/CREB1 and CREM/CREB1 heterodimers seem to bind the cAMP response element (CRE) sequence less potently than CREB1 homodimers (11, 12). Following cell activation, CREB proteins can be phosphorylated in their kinase-inducible domain; this allows for increased transactivation potential (13) and promotes the recruitment of cofactors such as CBP or p300 to the DNA polymerase complex (14, 15). In the case of CREB1 and ATF-1, this phosphorylation occurs on Ser133 and Ser63, respectively, and can be carried out by MAPKAP kinase-2, a kinase that acts immediately downstream of p38 MAPK (13).

In this study, we report on the expression, subcellular localization, phosphorylation, DNA-binding activity, and cytokine promoter occupancy of CREB transcription factors in human neutrophils stimulated by physiological agonists. We also demonstrate the involvement of CREB1 in cytokine production, the key role of its Ser133 residue for this response, and the upstream signaling events controlling CREB1 activation and that of another bZIP factor, C/EBPβ.
Materials and Methods

Abs and reagents

Abs raised against C/EBPβ (3082), phospho-CREB1 (9198, which also recognizes P-ATF1), phospho-C/EBPβ (3084), MSK1 (3489), and P-MSK1 (9595) were from Cell Signaling Technology (Beverly, MA). Abs against CREB1 (sc-186 and sc-240), CREB2 (sc-200), CREM (sc-34024), ATF-1 (sc-243), c-Jun (sc-1694), JunD (sc-74), and c-Fos (sc-52) were from Santa Cruz Biotechnology (Santa Cruz, CA). Luciferase constructs under the control of the wild-type (WT) human IL-8 promoter, as well as a mutant in its CRE-like site (IL-8 mutCREL), were a gift from Dr. M. A. Labow (Novartis Institute for Biomedical Research, Cambridge, MA) (16). Similarly, luciferase constructs under the control of the WT human CCL4 promoter (MIP1b WT), as well as a mutant in its CRE-like site (MIP1b mutCREL), were provided by Dr. M. Reitz (University of Maryland School of Medicine, Baltimore, MD) (17). The luciferase construct driven by three consensus CRE sequences (pCRE-Luc), as well as plasmids encoding WT CREB1, the SI33A mutant form, and a dominant-negative form (K-CREB) were all from BD Clontech (Mountain View, CA). Ficollo-Paque, T4 polynucleotide kinase, and poly(dI-dC) were from GE Biosciences (Baie d’Urfe’, QC, Canada); radionucleotides were from NEN (Boston, MA). Endotoxin-free (<2 pg/ml) RPMI 1640 medium and FCS were from Visient (St. Bruno, QC, Canada). Recombinant human cytokines were from R&D Systems (Minneapolis, MN), and UltraPure LPS (from Escherichia coli 0111:B4) was from InvivoGen (San Diego, CA). Acetylated BSA, diisopropylphosphorothionate, culture-grade DMSO, N-formylmethionylphenylalanine, and PMSF were from Sigma-Aldrich (St. Louis, MO). The protease inhibitors, aprotinin, 4-(2-aminomethyl)benzenesulfonyl fluoride, leupeptin, and pepstatin A, were all from Roche (Laval, QC, Canada). The p90 RSK inhibitor, BI-D1870, was purchased from the University of Wisconsin–Madison (Madison, WI) (16). The luciferase construct driven by the CRE of the wild-type (WT) human IL-8 promoter, as well as a mutant in its CRE-like site (IL-8 mutCREL). For whole-cell samples, boiling sample buffer was added directly to cell pellets, which were briefly vortexed and placed in boiling water for an additional 5 min. Samples were then centrifuged (2800 g, 10 min), resuspended at 2 × 106/ml in RPMI 1640 medium supplemented with 5% FCS, and laid to rest for 2.5 h. Cells were then cultured in the presence or absence of stimuli for 1 h (mRNA analyses) or 6 h (cytokine secretion and luciferase assays). Luciferase assays were conducted as described previously (6).

Cell isolation and culture

Neutrophils were isolated from the peripheral blood of healthy donors, following a protocol that was duly approved by an institutional ethics committee. The entire procedure was carried out at room temperature and under endotoxin-free conditions, as described previously (18). Purified neutrophils were resuspended in RPMI 1640 medium supplemented with 10% autologous serum at a final concentration of 5 × 105 cells/ml (unless otherwise stated). As determined by Wright staining and FACS analysis, the final neutrophil suspensions contained less than 0.2% monocytes or lymphocytes; neutrophil viability exceeded 98% after up to 4 h in culture, as determined by trypan blue exclusion and by Annexin V/PI FACS analysis. The PLB-985 cell line (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was cultured at 37˚C under a humidified 5% CO2 atmosphere, in the presence or absence of stimuli. After a 5-min incubation, the cells were nucleofected using the Amaxa (Königshofen, Germany) (19). The luciferase construct driven by three consensus CRE sequences (pCRE-Luc), as well as plasmids encoding WT CREB1, the SI33A mutant form, and a dominant-negative form (K-CREB) were all from BD Clontech (Mountain View, CA). Ficollo-Paque, T4 polynucleotide kinase, and poly(dI-dC) were from GE Biosciences (Baie d’Urfe’, QC, Canada). All other reagents were of the highest available grade, and all buffers and solutions were prepared using pyrogen-free clinical-grade water.

Immunobots

Cells were incubated at 37˚C in the presence or absence of stimuli. Incubations were stopped by adding equivalent volumes of ice-cold PBS supplemented with diisopropylphosphorothionate (final concentration, 2 mM) and phosphatase inhibitors (10 mM NaF, 1 mM Na2VO4, and 10 mM Na3P04). For whole-cell samples, boiling sample buffer was added directly to cell pellets, which were briefly vortexed and placed in boiling water for an additional 5 min. Samples thus prepared were sonicated to disrupt chromatin and stored at −20˚C prior to analysis. When cytoplasmic and nuclear fractions were prepared, incubations were stopped as described above, and neutrophils were disrupted by nitrogen cavitation as described previously (19, 20). Samples were electrophoresed, transferred onto nitrocellulose, and processed for immunoblot analysis as described previously (19).

EMSAs

Cells were incubated as described above, and nuclear extracts were prepared using a nitrogen cavitation procedure and analyzed in EMSA as described previously (21).

Real-time PCR analyses

Procedures and primers used are as described previously (7).
Inducible phosphorylation of CREB proteins in human neutrophils and signaling molecules involved

Phosphorylation of CREB1 on Ser\(^{133}\), and of ATF-1 on Ser\(^{63}\), reportedly leads to increased promoter activation potential (13). As shown in Fig. 1C, both CREB1 and ATF-1 were transiently phosphorylated on these residues in response to LPS or TNF-\(\alpha\); this effect was already evident after 5 min and was sustained for up to 1 h (depending on the donor). A much weaker signal was always detected in the case of P-ATF-1, however, indicating that CREB1 is the major phosphorylated CREB protein among the two. Other TLR agonists or cytokines (namely, peptidoglycan, IL-1, and IL-18) were also able to elicit CREB phosphorylation in neutrophils (Supplemental Fig. 1). The ability of neutrophil stimuli to phosphorylate CREB1 was not mediated by changes in intracellular cAMP levels, because they failed to modulate constitutive cAMP levels in neutrophils (Supplemental Fig. 2A), in agreement with previous studies (24–26). Conversely, a cell-permeable cAMP analog only modestly affected CREB phosphorylation in neutrophils, in comparison with the strong effect of TNF-\(\alpha\) on this response (Supplemental Fig. 2B).

**FIGURE 1.** Expression, distribution, and activation of CREB proteins in human neutrophils. (A) Freshly isolated neutrophils were either left untreated or stimulated for the indicated times with 300 ng/ml LPS. Reactions were stopped, and cells were boiled in sample buffer and processed for immunoblot analysis of their CREB proteins; 5 \(\times\) 10⁵ cells were loaded per lane. Samples were also immunoblotted for actin (as loading control). (B) Neutrophils were treated as described in (A) and disrupted by nitrogen cavitation; the resulting cytoplasmic and nuclear fractions processed for immunoblot analysis of their CREB content (\(\sim\)10⁶ cell equivalents/lane, representing 40 and 20 \(\mu\)g protein/lane for cytoplasmic and nuclear fractions, respectively). For comparison, samples were also immunoblotted for actin (loading control) and histone H3 (a nuclear marker). (C) Neutrophils were treated as described in (A); the cells were boiled in sample buffer and processed for immunoblot analysis using Abs raised against either phospho-CREB1 (Ser\(^{133}\)) and phospho-ATF-1 (Ser\(^{63}\)), or against CREB1 (loading control). Experiments shown in this figure are representative of at least three.

**FIGURE 2.** Induction and identity of CRE-binding activities in human neutrophils. (A) Neutrophils were either left untreated or stimulated for the indicated times with 300 ng/ml LPS. Cells were disrupted by nitrogen cavitation, and nuclear extracts were prepared and analyzed in EMSA (3 \(\mu\)g/lane) using a CRE oligonucleotide probe. (B) Nuclear extracts from LPS-treated neutrophils (300 ng/ml, 30 min) were incubated in the absence (“−”) or presence of various Abs, a 10-fold excess of unlabeled competitor oligonucleotide (“cold”), or a 25-fold excess of unlabeled mutant competitor oligonucleotide (“mut”), prior to the addition of labeled CRE probe, and subsequent EMSA analysis. Experiments shown in this figure are representative of at least three. Arrowheads indicate the specific CRE-binding activities.
We next investigated which upstream kinases might account for inducible CREB1 and ATF-1 phosphorylation. Because protein kinase (PK) A was the first kinase implicated in CREB phosphorylation, at least in vitro, we pretreated neutrophils with the PKA inhibitor, H89, prior to stimulation. As shown in Fig. 3A, the inhibitor had little or no effect on CREB1 phosphorylation. We then performed similar experiments with inhibitors of several kinases (i.e., p38 MAPK, MEK, PI3K/Akt, PKC, and RSK). As shown in Fig. 3B, inducible CREB1 and ATF-1 phosphorylation was only impaired by pretreatment with the p38 MAPK inhibitor. The effect of p38 MAPK inhibition was consistently observed across all blood donors, although the extent of the inhibition varied, being sometimes more pronounced (as seen for instance in Fig. 4A).

We also attempted to identify the kinase acting downstream from p38 MAPK. A promising candidate was MSK1, which can reportedly phosphorylate CREB1 on Ser133 and ATF-1 on Ser63 (27, 28). As shown in Fig. 3C, MSK1 is transiently activated in neutrophils, as illustrated by its phosphorylation on Thr581 (29), which peaked at 30 min—just like that of CREB1 and ATF-1, suggesting that these processes may be related. In support of this view, subcellular localization experiments revealed that MSK1 activation takes place in the nucleus (Fig. 3D), like that of CREB1 and ATF-1. Similarly, MSK1 activation was prevented by inhibitors of p38 MAPK but not MEK (Fig. 3E), as was the phosphorylation of CREB proteins. Finally, neutrophil pretreatment with a MSK1 inhibitor (Ro-31-8220) prevented inducible CREB phosphorylation (Fig. 4A) as well as inducible CRE-binding activity in EMSA (Fig. 4C). Noteworthy is that the effects of Ro-31-8220 on these responses mirrored those of the p38 MAPK inhibitor, SB202190 (Fig. 4A–C). These results indicate that MSK1 mediates (at least partially) the activation of CREB1 and ATF-1 by p38 MAPK.

We previously reported that C/EBPβ phosphorylation is essential for its involvement in cytokine production in human granulocytes, and that this was accompanied by an increased association of phosphorylated C/EBPβ to the CXCL8 promoter in neutrophils (7). We therefore investigated whether phosphorylated CREB1 is similarly recruited to chemokine promoters as well as the effect of p38 MAPK and MSK1 inhibition on this process. As shown in Fig. 5, CREB1 is constitutively associated to chemokine promoters in neutrophils, as previously shown for C/EBPβ and C/EBPε (7). Following cell stimulation with either LPS or TNF-α, there was sometimes a modest increase in the association of CREB1 or C/EBPβ to chemokine promoters (Fig. 5). Recruitment of both factors was much more pronounced when their phosphorylation is essential for their association to chemokine promoters.

**FIGURE 3.** Signaling components acting upstream of CREB activation in human neutrophils. (A) Neutrophils were pretreated (30 min, 37˚C) with 10 μM H89 prior to a 15-min incubation in the absence (“ctrl”) or presence of 300 ng/ml LPS. Cells were then processed for immunoblot detection of their phospho-CREB content; the same samples were also migrated on a parallel gel and immunoblotted for CREB1 as a loading control. (B) Neutrophils were pretreated (30 min, 37˚C) with various kinase inhibitors: 1 μM SB202190 (“SB”), 20 μM U0126 (“U0”), 20 μM PD98059 (“PD”), 10 μM LY-294002 (“LY”), 5 μM GF109203X (“GF”), or 10 μM BI-D1870 (“BID”). The cells were then incubated for 15 min in the absence (“ctrl”) or presence of 300 ng/ml LPS and processed for immunoblot detection of phospho-CREB; the same samples were migrated on a parallel gel and immunoblotted for CREB1 as a loading control. (C) Neutrophils were incubated in the absence (“ctrl”) or presence of 300 ng/ml LPS for the indicated times and processed for immunoblot analysis of their phospho-MSK1 (Thr581) content; the same samples also were migrated on a parallel gel and immunoblotted for CREB1 as a loading control. (D) Neutrophils were incubated as described in (C) and processed for immunoblot analysis of phospho-MSK1 and MSK1 (loading control). (E) Neutrophils were pretreated (30 min, 37˚C) with 1 μM SB202190 (“SB”) or 20 μM U0126 (“U0”), prior to a 15-min incubation in the absence (“ctrl”) or presence of 300 ng/ml LPS. Cells were then processed for immunoblot analysis of phospho-MSK1 and MSK1 (loading control). Experiments shown in this figure are representative of at least three.
phosphorylated forms were considered, and this response was strongly hindered by either p38 MAPK or MSK1 inhibition (Fig. 5). Thus, the p38-MSK1 signaling cascade controls the association of activated/phosphorylated bZIP factors to chemokine promoters.

The above results suggested an involvement for MSK1 in cytokine generation. Accordingly, pharmacological blockade of either p38 MAPK or MSK1 profoundly inhibited the inducible expression (Fig. 6A) and release (Fig. 6B) of inflammatory cytokines in LPS-stimulated neutrophils, in keeping with our previously published data showing that this functional response is under the control of the p38 MAPK pathway in these cells (5, 30).

**Functional impact of CREB factors on the promoter transactivation and gene expression of inflammatory cytokines**

Because p38 MAPK or MSK1 inhibition does not make it possible to discriminate between the potential effects of CREB and C/EBP factors, alternative approaches were undertaken to study the role of CREB factors in cytokine generation by neutrophils. To this end, we first determined whether LPS or TNF-α stimulation can trans-activate a CREB-driven luciferase construct in human neutrophil-like PLB-985 cells, whose cytokine expression pattern closely matches that of primary neutrophils (6). As shown in Fig. 7A, both stimuli promoted the activity of this construct, in good agreement with the effect of LPS and TNF-α on CREB protein phosphorylation in primary neutrophils. We next investigated the effect of mutating CREB binding sites within actual chemokine promoters. For this purpose, we took advantage of the CXCL8 and CCL4 promoters, which both feature CRE-like sequences within their proximal promoters (16, 17). Neutrophil-like PLB-985 cells were nucleofected with luciferase constructs driven by WT CXCL8 and CCL4 promoters or by variants of these promoters featuring mutated CRE-like sequences, prior to cell stimulation. As shown in Fig. 7B, both chemokine promoters were strongly activated by LPS or TNF-α, and mutation of their CRE-like sequences abolished promoter induction. Noteworthy is that CRE mutations also affected the basal activity of chemokine promoters (Fig. 7B). Thus, CREB factors appear to play an important role in the context of CXCL8 and CCL4 promoter transactivation in human neutrophil-like PLB-985 cells.

To exclude the possibility that unrelated transcription factors might bind the CRE-like sites within the proximal CXCL8 and CCL4 promoters, WT CREB1, a dominant-negative CREB1 mutant (K-CREB), and a point mutant in which Ser133 had been mutated to alanine (S133A) were overexpressed into human neutrophil-like PLB-985 cells, and following stimulation with LPS or TNF-α, cytokine production was determined. As shown in Fig. 8, over-expression of either K-CREB or CREB (S133A) strongly inhibited the generation of CXCL8, CCL3, CCL4, IL-1β, and TNF-α in neutrophil-like PLB-985 cells. Therefore, phosphorylation of CREB1 on Ser133 seems to be essential for the production of several inflammatory cytokines in human granulocytes.

**Discussion**

Neutrophils influence innate and adaptive immunity by generating numerous cytokines and chemokines (1–3). We have previously shown that this response is largely dependent on transcription factors typically associated with inflammation, such as NF-κB and C/EBP factors (5–7). By contrast, AP-1 factors do not seem to play any significant role (6, 8, 9). In this study, we show that the CREB1 transcription factor also participates in this important functional response of neutrophils, and we additionally shed light on the upstream signaling intermediates controlling both CREB and C/EBP factors.

We first investigated which CREB family members are expressed in neutrophils. We had previously reported that CREB1 is a major component of nuclear complexes that bind to an AP-1 probe in human neutrophils (8). We now report that in addition to CREB1, ATF-1 is also strongly expressed in neutrophils, whereas only low amounts of CREM are detectable. These proteins were primarily nuclear in neutrophils and were rapidly and transiently phosphorylated in response to cytokines or TLR agonists. The phosphorylation of CREB1 and ATF1 was accompanied by an increase in the specific binding of two nuclear complexes to a CRE-containing oligonucleotide probe in EMSA. Supershift analyses revealed that CREB1 is a constituent of both complexes, whereas ATF-1 was only present in the faster-migrating one. Finally, we showed that CREB1 is constitutively associated with the proximal promoter region of several chemokines (CXCL8, CCL3, and CCL4), as we...
had previously observed in the case of other bZIP factors (e.g., C/EBPβ and C/EBPε) (7). Neutrophil stimulation led to the recruitment of P-CREB1 to the same chemokine promoters, as we had shown previously in the case of P-C/EBPβ in the context of the CXCL8 promoter (7). It is worth pointing out that the Ab we used to immunoprecipitate P-CREB1 in these ChIP assays also recognizes P-ATF-1 (according to the manufacturer and as seen in Fig. 3). Thus, it is possible that a part of the signal amplified in these ChIP assays reflects the recruitment of P-ATF-1 to the CXCL8, CCL3, and CCL4 promoters, in addition to that of P-CREB1. This being said, the proportion attributable to ATF-1 is arguably a fairly minor one, because the Ab consistently detected much more P-CREB1 than P-ATF-1 in immunoblots of activated neutrophils (Fig. 3). Our overexpression experiments using a CREB1 point mutant (S133A)
From a more functional standpoint, the phosphorylation of CREB proteins and their association to chemokine promoters suggested a role in transcriptional activation. Accordingly, we observed that inducers of CREB protein phosphorylation (i.e., LPS and TNF-α) also stimulated the activity of a CREB-driven luciferase construct in human neutrophil-like PLB-985 cells. Moreover, the use of proximal chemokine promoter variants mutated in their CRE-like sequences clearly demonstrated that these binding sites are crucial for CXCL8 and CCL4 promoter transactivation in human granulocytes. Overexpression of a dominant-negative CREB mutant (K-CREB) or of a CREB1 point mutant (S133A) established that CREB proteins, and in particular CREB1 (via its Ser133 residue), play a crucial role in the inducible expression of several inflammatory cytokines (CXCL8, CCL3, CCL4, IL-1β, and TNF-α) in human granulocytes. Noteworthy is that the same cytokines were previously found to depend on CREB1 and C/EBP transcription factors as well (5–7) but not on the AP-1 factor (6, 8, 9). One notable exception is the TNF-α gene, whose expression did not depend on C/EBP (7). As a result, the current study yields a clearer picture of how cytokine expression is regulated in neutrophils. In a broader context, our present findings are consistent with observations made in other cell types, showing that CREB proteins are required for the inducible expression of CXCL8 and IL-1β in human monocytic cell lines (31, 32) and for expression of both CCL4 and TNF-α in human T cells (17) and murine macrophages (33, 34). In contrast, a role for CRE sequences within the human CCL3 proximal promoter had been reported using human T cell lines (35), but the transcription factors mediating this phenomenon had not been identified. To our knowledge, our data represent the first demonstration of a role for CREB1 in controlling human CCL3 gene expression.

FIGURE 6. Effect of p38 MAPK and MSK1 inhibition on inflammatory cytokine expression and release in human neutrophils. (A) Neutrophils were pretreated (30 min, 37°C) with 1 μM SB202190 (“SB”), 5 μM Ro318220 (“Ro”), or diluent control (0.1% DMSO). After an additional 30-min incubation in the absence (“ctrl”) or presence of 300 ng/ml LPS or 100 U/ml TNF-α, RNA was extracted and processed for quantitative PCR analysis. Values were normalized over 18S and are represented as fold increase relative to unstimulated cells. (B) Neutrophils were preincubated as described in (A) and cultured for 8 h in the absence (“ctrl”) or presence of 300 ng/ml LPS or 100 U/ml TNF-α, prior to ELISA analysis of culture supernatants. Data are mean ± SEM from at least three independent experiments. **p < 0.01 versus stimulus alone.

also lend support to this view. Taken together, the above results offer a detailed appreciation of CREB protein expression and activation in human neutrophils.

From a more functional standpoint, the phosphorylation of CREB proteins and their association to chemokine promoters suggested a role in transcriptional activation. Accordingly, we observed that inducers of CREB protein phosphorylation (i.e., LPS and TNF-α) also stimulated the activity of a CREB-driven luciferase construct in human neutrophil-like PLB-985 cells. Moreover, the use of proximal chemokine promoter variants mutated in their CRE-like sequences clearly demonstrated that these binding sites are crucial for CXCL8 and CCL4 promoter transactivation in human granulocytes. Overexpression of a dominant-negative CREB mutant (K-CREB) or of a CREB1 point mutant (S133A) established that CREB proteins, and in particular CREB1 (via its Ser133 residue), play a crucial role in the inducible expression of several inflammatory cytokines (CXCL8, CCL3, CCL4, IL-1β, and TNF-α) in human granulocytes. Noteworthy is that the same cytokines were previously found to depend on the NF-κB and C/EBP transcription factors as well (5–7) but not on the AP-1 factor (6, 8, 9). One notable exception is the TNF-α gene, whose expression did not depend on C/EBP (7). As a result, the current study yields a clearer picture of how cytokine expression is regulated in neutrophils. In a broader context, our present findings are consistent with observations made in other cell types, showing that CREB proteins are required for the inducible expression of CXCL8 and IL-1β in human monocytic cell lines (31, 32) and for expression of both CCL4 and TNF-α in human T cells (17) and murine macrophages (33, 34). In contrast, a role for CRE sequences within the human CCL3 proximal promoter had been reported using human T cell lines (35), but the transcription factors mediating this phenomenon had not been identified. To our knowledge, our data represent the first demonstration of a role for CREB1 in controlling human CCL3 gene expression.

Our investigation of the signaling events upstream of CREB protein activation revealed that this process is under the control of the p38-MSK1 cascade in neutrophils. This is consistent with the reported ability of MSK1 to mediate CREB1 and ATF-1 phosphorylation in other systems (27), and with the fact that CREB1 is a known MSK substrate (28). Importantly, we also found that C/EBPβ phosphorylation is similarly controlled, and that cytokine promoter occupancy by these phosphorylated bZIP factors is also regulated by p38 MAPK and MSK1. In view of our previous finding, that p38 MAPK activation is governed by TAK1 in human neutrophils (22), the above results show that transcriptional regulation of inflammatory cytokine production by CREB and C/EBP factors occurs downstream of the TAK1-p38-MSK1 signaling cascade in neutrophils. Our data also represent the first demonstration that of a role for MSK1 in any neutrophil response. Whereas MSK1 is known to be controlled by both p38 MAPK and the MEK/ERK module in some cell types and also to participate in regulating their proinflammatory cytokine expression (28, 36), we found that in neutrophils, MSK1 was unaffected by MEK inhibition, thereby revealing that its regulation can be cell type specific. Similarly, although RSK kinases can phosphorylate CREB1 in other cell types (37, 38), we found that the RSK inhibitor, BI-D1870, had no effect on CREB1 phosphorylation in neutrophils (Fig. 3B). Thus, it appears that the MEK-ERK-RSK cascade does not participate in CREB-mediated gene induction in LPS- and TNF-stimulated neutrophils. In keeping with this conclusion, we observed in this study that MEK inhibitors indeed failed to affect inducible CREB protein phosphorylation under these conditions. On a final note, it should be mentioned that the MSK1 inhibitor we used (Ro-31-8220) can potentially affect other kinases, such as PKA, PKC, and RSK1 (39). Because we found that inhibitors of PKA (H89), PKC (GF109203X), or RSK (BI-D1870) had no effect on CREB protein phosphorylation or DNA binding (Figs. 3B, 4B; data not shown), it seems reasonable to conclude that the effects of Ro-31-8220 observed in this study can be mainly attributed to MSK1 inhibition.

Collectively, our data considerably extend our knowledge of CREB protein expression in human neutrophils, of their activation in response to physiological stimuli, of their role in inflammatory
cytokine production by these cells, of the parallel activation of another bZIP factor, and of the upstream signaling machinery controlling both CREB and C/EBP factors. As this paper was being completed, Dumitru et al. (40) reported that supernatants from head- and-neck cancer cell lines promote CREB1 Ser133 phosphorylation in neutrophils, and that this was inhibited by high concentrations (10 μM) of SB202190. Moreover, a 72-h culture of DMSO-differentiated HL-60 cells with CREB small interfering RNA inhibited CXCL8, CCL4, and matrix metalloproteinase-9 release elicited by a 24-h exposure to these cancer cell line supernatants (40). Although

**FIGURE 7.** Induction of CRE-driven transcription and role of CRE sequences on chemokine promoter activity in neutrophil-like PLB-985 cells. (A) Granulocytic PLB-985 cells (day 5) were nucleofected with luciferase constructs driven by three repeats of the consensus CRE sequence. The cells were then cultured for 4 h in the absence ("ctrl") or presence of 300 ng/ml LPS or 100 U/ml TNF-α, prior to cell lysis and luciferase activity measurement. Mean ± SEM of four (LPS) and three (TNF) independent experiments. (B) Granulocytic PLB-985 cells (day 5) were nucleofected with luciferase constructs driven by the WT CXCL8 ("IL-8 WT") or CCL4 ("MIP1b WT") proximal promoters or by versions of these promoters that were mutated in their CRE-like sites ("IL-8 mutCREL" and "MIP1b mutCREL"). The cells were then cultured for 4 h at 37°C in the presence or absence of 300 ng/ml LPS or 100 U/ml TNF-α, prior to cell lysis and luciferase activity measurement. Mean ± SEM from at least three independent experiments. **p < 0.001.

**FIGURE 8.** Effect of overexpressed CREB1 variants on cytokine production in neutrophil-like PLB-985 cells. Granulocytic PLB-985 cells (day 5) were nucleofected with a plasmid encoding wild-type CREB1, a dominant negative CREB1 (K-CREB), a CREB1 point mutant (S133A), or empty vector (pCMV). After a 4-h culture, samples were taken and processed for immunoblot analysis of overexpressed proteins (inset). The remainder of the cells were cultured for 6 h in the absence ("unstim") or presence of 300 ng/ml LPS or 100 U/ml TNF-α, prior to ELISA analysis of culture supernatants. The experiment depicted is representative of three.
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Figure S1
A

![Bar graph showing cAMP levels](image)

B

![Western blot analysis](image)

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Figure S2
Figure S1. Phosphorylation of CREB1 by cytokines and a TLR agonist in human neutrophils. Freshly isolated neutrophils were stimulated for 10 min with 5 µg/ml peptidoglycan (“PGN”), 10 ng/ml IL-1β, 100 ng/ml IL-18, or left unstimulated (“ctrl”). Reactions were stopped, and cells were boiled in sample buffer and processed for immunoblot analysis using antibodies raised against either phospho CREB1 (Ser133) or CREB1 (loading control). The experiment shown is representative of three.

Figure S2. Effect of neutrophil stimulation on intracellular cAMP levels, and of cAMP on CREB phosphorylation. (A) Neutrophils were stimulated for the indicated times with 300 ng/ml LPS, 100 U/ml TNFα, 10 µM forskolin (“forsk”), or left unstimulated (“ctrl”), prior to determination of their intracellular cAMP concentration. Mean ± s.e.m. from 3 independent experiments. *, p<0.01. (B) Neutrophils were stimulated for the indicated times with 100 U/ml TNFα, 100 µM or 1 mM dibutyryl cAMP, 100 nM prostaglandin E2 (“PGE”), or left unstimulated (“ctrl”); the cells were then boiled in sample buffer and processed for immunoblot analysis using antibodies raised against either phospho CREB1 (Ser133) or CREB1 (loading control). The experiment shown is representative of three.