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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 TNFα 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 cascade, 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.
Materials and Methods

Abs and reagents

Abs raised against C/EBPβ (3082), phospho-CREB1 (9198, which also recognizes P-ATF-1), phospho-C/EBPβ (3084), MSK1 (3489), and P-MSK1 (9595) were from Cell Signalling 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 S133A mutant form, and a dominant-negative form (K-CREB) were all from BD Clontech (Mountain View, CA). Ficoll–Paque, T4 polynucleotide kinase, and poly(dI-dC) were from GE Biosciences (Baille d’Urfé, QC, Canada); radiolucentides were from NEN (Boston, MA). Endotoxin-free (≤2 pg/ml) RPMI 1640 medium and FCS were from Vivasent (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, diisopropylfluorophosphate, culture-grade DMSO, N-formylmethionyl-phenylalanine, 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 Dundee (Dundee, Scotland). The other kinase inhibitors were all purchased through Cedarslane Labs (Mississauga, ON, Canada). All other reagents were of the highest available grade, and all buffers and solutions were prepared using pyrogen-free clinical-grade water.

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 × 10⁶ 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/or inhibitors, for the indicated times. Culture supernatants were carefully collected, snap-frozen in liquid nitrogen, and stored at −70°C. Samples were analyzed in ELISA using commercially available capture and detection Ab pairs (R&D Systems, BD Biosciences).

Transient transfections and luciferase assays.

PLB-985 granulocytes were nucleofected mostly as described previously (6). Briefly, 10⁶ cells were pelleted and resuspended in 100 μl of a home-made nucleofection buffer (25 mM HEPES [pH 7.6], 120 mM KCl, 2 mM MgCl₂, 10 mM K₃HPO₄, and 5 mM t-cysteine), and 5 μl phosphatidylserine was added. After a 5-min incubation, the cells were nucleofected using the Amaxa 001 setting of a Nucleofector instrument (Amarsa Biosystems). Cells were then centrifuged (280 × 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).

Chromatin immunoprecipitation assays

Procedures and primers used are essentially as described previously (7).

Data analysis

All data are represented as the mean ± SEM of at least three independent experiments. Statistical differences between groups were analyzed by one-way ANOVA, followed by Bonferroni’s post hoc test, using Prism 5 software (GraphPad Software, San Diego, CA).

Results

Expression and distribution of CREB proteins in human neutrophils

To determine which CREB isoforms are expressed by neutrophils, unstimulated cells were boiled in sample buffer and processed for immunoblotting using isospecific Abs. As shown in Fig. 1A, resting neutrophils express CREB1, as we already reported (7), but also ATF-1 and, somewhat weakly, CREM as well. Neutrophil stimulation with LPS or TNF-α did not significantly alter the cellular pool of any of these proteins for up to 4 h (Fig. 1A; data not shown). We next examined the subcellular distribution of the CREB proteins. Neutrophils were disrupted by nitrogen cavitation, and the resulting nuclear and cytoplasmic fractions were analyzed by immunoblot; we previously showed that this fractionation procedure yields cytoplasmic fractions and intact nuclei that are exempt from cross-contamination (18, 19, 22). As shown in Fig. 1B, all CREB proteins are primarily nuclear. Cell stimulation with LPS, peptidoglycan, TNFα, IL-1β, or IL-18 did not alter their cellular distribution (Fig. 1B; data not shown).

Detection of CREB DNA-binding activities in human neutrophils

We next determined whether neutrophil stimulation promotes the binding of nuclear CREB proteins to their cognate DNA sequence, the CRE. As shown in Fig. 2A, cell stimulation rapidly and transiently induced CREB DNA-binding activities. The inducible CRE-binding complexes were specific, because they were competed out by a 10-fold excess of unlabeled probes but unaffected by a 25-fold excess of unlabeled oligonucleotides in which the DNA-binding sequence was mutated (Fig. 2B). In contrast, neither CREM nor CREB2 was present in either complex (Fig. 2B). Because Jun/Fos proteins can bind to CRE sequences (23), we also investigated whether they may participate in CRE-binding complex formation in neutrophils. However, none of the Jun/Fos proteins investigated were constituents of the complexes (Fig. 2B).
Inducible phosphorylation of CREB proteins in human neutrophils and signaling molecules involved

Phosphorylation of CREB1 on Ser133, and of ATF-1 on Ser63, 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-α; 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-α 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 × 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 protein content (~10⁶ cell equivalents/lane, representing 40 and 20 μg 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 (Ser133) and phospho–ATF-1 (Ser63), 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 μg/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 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 Ser\(^{133}\) and ATF-1 on Ser\(^{65}\) (27, 28). As shown in Fig. 3C, MSK1 is transiently activated in neutrophils, as illustrated by its phosphorylation on Thr\(^{581}\) (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.

To determine whether MSK1 might affect other transcription factors beside CREB family proteins, we focused on two such factors that are known to mediate cytokine gene expression in neutrophils (i.e., members of the C/EBP and NF-κB families) (5–7). For comparison purposes, we also examined the effect of inhibiting the p38 MAPK and MEK pathways, which participate in cytokine production in neutrophils (5, 30). As shown in Fig. 4D, inhibitors of both MSK1 and p38 MAPK potently inhibited the inducible phosphorylation of C/EBP\(\beta\), whereas inhibition of the MEK pathway had no effect on this response. By contrast, none of these inhibitors had any effect on RelA phosphorylation or IkB-α degradation (data not shown), as we already reported in the case of p38 MAPK and MEK inhibition (5). Thus, the p38/MSK1 axis governs the phosphorylation of both CREB and C/EBP factors in neutrophils but not that of IκB kinase/NF-κB pathway components.

We previously reported that C/EBP\(\beta\) phosphorylation is essential for its involvement in cytokine production in human granulocytes, and that this was accompanied by an increased association of phosphorylated C/EBP\(\beta\) 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/EBP\(\beta\) and C/EBP\(\gamma\) (7). Following cell stimulation with either LPS or TNF-α, there was sometimes a modest increase in the association of CREB1 or C/EBP\(\beta\) to chemokine promoters (Fig. 5). Recruitment of both factors was much more pronounced when their

**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 also 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 (Thr\(^{581}\)) content; the same samples also were migrated on a parallel gel and immunoblotted for MSK1 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.

Functional consequences of p38 MAPK and MSK1 inhibition on other transcriptional processes and cytokine generation in human neutrophils

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Like PLB-985 cells, CXCL8 and CCL4 promoter transactivation in human neutrophil-affected the basal activity of chemokine promoters (Fig. 7B). Thus, phospho-CRE-like sequences, prior to cell stimulation. As shown in Fig. 7A, both chemokine promoters were strongly activated by phosphorylated 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 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 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 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-α-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 PKC, PKA, and RSK1 (39). Because we found that inhibitors of PKA (H89), PKC (Golf9203X), 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 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), also stimulate the activity of a CREB-driven luciferase construct in human neutrophil-like PLB-985 cells. Moreover, the use of
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 mutXPFL” 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.
it is not clear how a prolonged CREB knockdown might affect the granulocytic differentiation process in HL-60 cells, and how this may in turn influence the release of inflammatory mediators, the data are nevertheless in full agreement with the results presented in this paper. Conversely, although the soluble mediators activating CREB that are present in cancer cell line supernatants were not identified, the data presented in this study suggest that inflammatory cytokines (such as TNF-α, IL-1β, or IL-18) could account for at least some of the observed CREB Ser133 phosphorylation, especially because all three cytokines can activate CREB proteins in neutrophils (as shown in this study). Whatever the case may be, it is becoming clear that CREB proteins, along with MSK1 and upstream signaling components, all represent potential therapeuetic targets that could be exploited in the context of several chronic inflammatory diseases that prominently feature neutrophils and their products.

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Disclosures

The authors have no financial conflicts of interest.

References