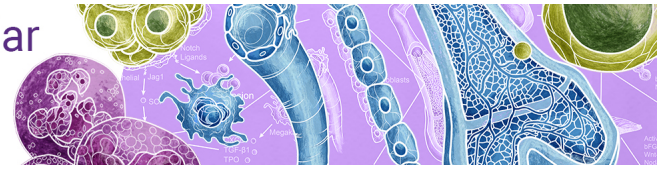

The Journal of Immunology

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Corticotropin-Releasing Factor and the Urocortins Induce the Expression of TLR4 in Macrophages via Activation of the Transcription Factors PU.1 and AP-1[1]

Christos Tsatsanis,[2,3]* Ariadne Androulidaki,[2]* Themis Alissafi,* Ioannis Charalampopoulos,[†] Erini Dermitzaki,* Thierry Roger,[‡] Achille Gravanis,[†] and Andrew N. Margioris[3]*

Corticotropin-releasing factor (CRF) augments LPS-induced proinflammatory cytokine production from macrophages. The aim of the present study was to determine the mechanism by which CRF and its related peptides urocortins (UCN) 1 and 2 affect LPS-induced cytokine production. We examined their role on TLR4 expression, the signal-transducing receptor of LPS. For this purpose, the murine macrophage cell line RAW 264.7 and primary murine peritoneal macrophages were used. Exposure of peritoneal macrophages and RAW 264.7 cells to CRF, UCN1, or UCN2 up-regulated TLR4 mRNA and protein levels. To study whether that effect occurred at the transcriptional level, RAW 264.7 cells were transfected with a construct containing the proximal region of the TLR4 promoter linked to the luciferase gene. CRF peptides induced activation of the TLR4 promoter, an effect abolished upon mutation of a proximal PU.1-binding consensus or upon mutation of an AP-1-binding element. Indeed, all three peptides promoted PU.1 binding to the proximal PU.1 site and increased DNA-binding activity to the AP-1 site. The effects of CRF peptides were inhibited by the CRF_2 antagonist anti-sauvagine-30, but not by the CRF_1 antagonist antalarmin, suggesting that CRF peptides mediated the up-regulation of TLR4 via the CRF_2 receptor. Finally, CRF peptides blocked the inhibitory effect of LPS on TLR4 expression. In conclusion, our data suggest that CRF peptides play an important role on macrophage function. They augment the effect of LPS by inducing *Tlr4* gene expression, through CRF_2, via activation of the transcription factors PU.1 and AP-1. *The Journal of Immunology,* 2006, 176: 1869–1877.

It is now well-established that corticotropin-releasing factor (CRF)[4] and its related peptides affect the inflammatory response in a paracrine/autocrine manner (1–8). CRF acts as an ad hoc proinflammatory factor because blockade of its effect by specific anti-CRF serum or CRF antagonists attenuates the inflammatory response in several models of inflammation including that of carrageenin, turpentine, and Gram-negative bacterial LPS-induced inflammation (9–11). A well-defined immune target of CRF is the mast cell (12–17). Indeed, mast cells express the CRF and the CRF-related protein urocortin (UCN) 1 and possess their specific binding sites (18). We have hypothesized that macrophages may represent an additional immune target of CRF and UCNs because UCN1 is also present at the sites of inflammation inducing IL-1 and IL-6 secretion from PBMC (7). Indeed, human peripheral blood monocytes, Kupffer cells, and monocytes/macrophages express specific CRF-binding sites (3, 19–21). Recent studies from our laboratory indicate that RAW 264.7 macrophages and primary, thioglycolate-elicited peritoneal macrophages isolated from C57BL/6 mice express both the CRF_1 and CRF_2 receptors and treatment with LPS partly affects the expression of CRF_1 but not that of CRF_2 (22). Moreover, we have found that CRF augments LPS-induced cytokine production by macrophages (11), thus increasing their sensitivity to LPS.

LPS transduces signals via TLR4 while forced overexpression of TLR4 results in increased cytokine secretion in response to LPS (23). Therefore, the aim of the present study was to examine the role of CRF and its related peptides UCN1 and UCN2 on the regulation of TLR4 expression by macrophages. TLR4 is the principal mediator of macrophage response to LPS (24). TLR4 expression is primarily regulated by the transcription factor PU.1 (25). Recent experimental evidence indicate that AP-1 is also critical for TLR4 expression together with a distal-binding site of an Ets family protein and a GATA-like binding element (26). PU.1 belongs to the Ets family of transcription factors and is expressed primarily on macrophages and cells of myeloid linage (27, 28). CRF has been reported to activate AP-1 in different cell systems including keratinocytes and chromaffin cells (29–31) with a complex composition of the transcription factor that implicated c-*fos*, JunB, Fra2, and JunD at different time points of CRF stimulation (30). However, no information is available on the role of UCN1 or UCN2 on AP-1-binding activity.

In the present study, we have examined the effect of the CRF peptides CRF, UCN1, and UCN2 and that of CRF_1 and CRF_2 synthetic antagonists on: 1) TLR4 mRNA and protein expression in the murine macrophage cell line RAW 264.7 and in freshly

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[4] Abbreviations used in this paper: CRF, corticotropin-releasing factor; UCN, urocortin; Ct, cycle threshold; m, mouse.

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isolated primary murine peritoneal macrophages; 2) the activation of the transcription factors PU.1 and AP-1, main regulators of TLR4 expression; 3) TLR4 promoter activity in RAW 264.7 cells transfected with the minimal TLR4 promoter containing the proximal promoter region linked to the luciferase gene; and 4) the inhibitory effect of LPS on TLR4 expression.

Materials and Methods

Cell culture

The murine macrophage cell line RAW 264.7 and freshly isolated primary murine peritoneal macrophages have been used. RAW 264.7 cells were obtained from the American Type Culture Collection. They were cultured in DMEM supplemented with 10% FCS, 10 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all purchased from Invitrogen Life Technologies), at 5% CO_2 and 37°C. Cells were plated in 6-well plates at a concentration of 4×10^5/ml 1 day before stimulation. Cells were then stimulated with synthetic rat/human CRF (Sigma-Aldrich) at a concentration of 10^{-8} M, synthetic rat UCN1 (Sigma-Aldrich) at a concentration of 10^{-8} M, synthetic mouse UCN2 (provided by Dr J. Spiess, University of Hawaii, Honolulu, HI) at 10^{-8} M, or 10 μg/ml *Escherichia coli*-derived LPS (serotype O111:B4, catalog no. L2630; Sigma-Aldrich), as previously used to determine the effects of CRF on proinflammatory cytokine expression (11). When inhibitors were used, cells were pretreated for 1 h within a 100-fold excess previously shown to inhibit CRF receptor signals (32), being anti-sauvagine-30 10^{-6} M (provided by Dr. J. Spiess) or antalarmin at 10^{-6} M (provided by Dr. G. Chrousos, University of Athens, Athens, Greece) before stimulation with CRF or UCNs.

Primary murine peritoneal macrophages were elicited by 4% thioglycolate prepared and autoclaved 2 days before administration. A total of 1.5 ml of the solution was injected i.p. in C57BL/6 mice and peritoneal macrophages were isolated by lavage of the peritoneal cavity with DMEM (11, 33). Cells were then cultured in DMEM supplemented with 10% FCS, 10 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen Life Technologies). Cells were plated at a concentration of 5×10^5/ml and maintained in culture for 24 h. The medium was replaced with DMEM that did not contain FCS 12 h before stimulation. The Animal Facility Committee of the University of Crete School of Medicine approved the experimental procedures described.

Isolation of total RNA and real-time RT-PCR

Total cellular RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies). cDNA was prepared by reverse transcription (Thermoscript RT; Invitrogen Life Technologies) and amplified by PCR using the following primers pairs: for β-actin, sense, 5′-TCA GAA GAA CTC CTA TGT GG-3′ and antisense, 5′-TCT CTT TGA TGT CAC GCA CG-3′, giving a 499-bp product; for mouse TLR4, sense, 5′-ACC AAT GCA TGG ATC AGA AA-3′ and antisense, 5′-GTC TCC ACA GCC ACC AGA TT-3′ resulting in a 295-bp product. A total of 1 μl of cDNA was used together with the primers shown above in a 20-μl reaction, using SYBR green as a marker for DNA content, provided in the SYBR Green PCR Master Mix (Applied Biosystems). Amplification was performed in an ABI PRISM 7000 Real-Time PCR apparatus for a maximum of 40 cycles as follows: 40 s at 94°C, 40 s at 53°C, 1 min at 72°C. No by-products were present in the reaction as indicated by the dissociation pattern provided at the end of the reaction and by agarose gel electrophoresis (data not shown). The amplification efficiency of the TLR4 product was the same as the one of β-actin as indicated by the standard curves of amplification, allowing us to use the formula: fold difference $= 2^{-(\Delta CtA - \Delta CtB)}$, where Ct is the cycle threshold. Reactions were performed in triplicate to allow for statistical evaluation. Each experiment was repeated three times.

FACS analysis

In brief, cells were washed twice with PBS containing 1% BSA. Then, anti-mouse TLR4-PE-conjugated Ab (clone MTS510; e-Bioscience) was added, and cells were incubated at 4°C for 20 min. Cells were washed twice with PBS containing 1% BSA and analyzed on a flow cytometer (FACSCalibur; BD Biosciences).

Confocal laser-scanning microscopy

Cells were grown in 8-well chamber slides (Costar). At the end of each incubation period, cells were fixed by exposure to 3.7% formaldehyde, permeabilized by 0.2% Triton for 10 min, washed, and 0.1% FCS was added for 15 min. Cells were incubated subsequently with rabbit polyclonal Ab against PU.1 (Santa Cruz Biotechnology) at 4°C followed by anti-rabbit FITC-conjugated secondary Ab (Sigma-Aldrich). The cover slips were analyzed using a confocal laser-scanning module (Leica Lasertechnik) attached to an inverted microscope (Zeiss IM35) equipped with an argon-krypton ion laser. Confocal images were acquired using a 63/1.25 oil immersion objective and dedicated confocal laser-scanning microscopy software (Leica Lasertechnik).

EMSA

Nuclear extracts of RAW 264.7 cells treated with the three peptides at 10^{-8} M for 1 h were isolated as previously described (33, 34). Briefly, cells were resuspended in a lysis buffer containing 0.6% Nonidet P-40, 10 mM HEPES, 10 mM KCl, 0.2 mM EDTA to extract the cytoplasmic proteins. The nuclei were then lysed in 20 mM HEPES, 25% glycerol, 0.4 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors (Complete) by rocking for 15 min at 4°C. The protein in the nuclear lysates was quantified using the Bradford assay (Bio-Rad). Seven micrograms of nuclear extracts were incubated with 5×10^5 cpm of a ^{32}P-labeled dsDNA probe representing the proximal PU.1-binding site of the TLR4 promoter (Ets^P: AGC CAGCTTCCTCTTGCTGTTCC) or an oligonucleotide containing a mutation at the PU.1 binding site of (mouse (m) Ets^P: AAAGCCA GCTAGTTCTTGCTGTTCC). For the AP-1-binding assays, nuclear protein extracts were incubated with an oligonucleotide corresponding to the AP-1-binding site present in the TLR4 promoter (AGAGGTCAGAT GACTTCCTGGGATCA) and, as a control, with an oligonucleotide containing a mutation at the AP-1-binding site (GCCCAGAGGTCAGACCACT TCCTGGG). Incubation was conducted for 30 min on ice in a binding buffer containing 20 mM HEPES (pH 7.5), 0.5 mM EDTA, 5 mM $MgCl_2$, 50 μg/ml BSA, 0.005% Nonidet P-40, 60 mM KCl, 10 mM DTT, 10% glycerol, and 1.5 μg of poly(dI/dC). The protein-bound probe was separated from the unbound by electrophoresis in a 6% nondenaturing polyacrylamide gel. To determine the specificity of the complex, competition experiments were performed by incubating the extracts with the labeled probe in the presence of 5- to 100-fold excess of unlabeled oligonucleotide. To verify that the electrophoretic mobility shift observed was due to the PU.1 protein, 5 μl of an anti-PU.1 Ab (Santa Cruz Biotechnology) were incubated with the nuclear extract for 1 h on ice before addition of the labeled oligonucleotide.

Transfections and luciferase assay

The minimal TLR4 promoter containing 550 bp of the proximal promoter region linked to the luciferase gene (pGL3 mTLR4E), provided by Dr. M. Rehli (University of Regensburg, Regensburg, Germany), was transfected in RAW 264.7 cells by electroporation. In parallel experiments, the plasmid pGL3 mTLR4 (−518 to +223) was used and the same construct that carries mutations either at the PU.1 site (mEts^P TLR4; Ref. 26) or at the AP-1 site (26) was used. Briefly, 0.8 ml of cells at a concentration of 5×10^6 cells/ml in hypotonic electroporation buffer (Eppendorf) were transferred in electroporation cuvettes (4-mm gap) and subjected to four pulses at 570 V using an Eppendorf Multiporator. Cells were then plated in DMEM containing 10% FBS for 4 h. Cells were then washed and incubated with growth medium containing the corresponding peptides for 18 h. Cells were lysed and luciferase activity was measured according to the manufacturers' instructions (BD Biosciences). Cell viability following transfection was estimated by trypan blue staining and found to be the same in all treatments. Each transfection was performed in triplicate to allow statistical evaluation and control for possible variations in transfection efficiency. Results are representative of three independent experiments.

Statistical analysis

For the statistical evaluation, we used ANOVA post hoc comparison of means followed by two multiple comparison tests: the Fisher's least significance difference and the Newman-Keuls test. For data expressed as percent changes or fold difference, we used the nonparametric Kruskal-Wallis test for several independent samples.

Results

CRF peptides induce the expression of TLR4 in primary murine macrophages and the RAW 264.7 cell line

The effect of the CRF peptides on TLR4 protein on the surface of macrophages was examined by flow cytometric analysis. Compared with TLR4 levels in untreated cells ("control" panel), treatment with CRF, UCN1, or UCN2, at a concentration of 10^{-8} M for 24 h, increased TLR4 expression on the murine macrophage

cell line RAW 264.7 (Fig. 1*A*), and on freshly isolated thioglycolate-elicited primary murine peritoneal macrophages (Fig. 1*B*). Specifically, CRF, UCN1, and UCN2 induced a 2.2-, 1.9-, and 2.3-fold increase of TLR4 expression on RAW 264.7 macrophages, and a 1.7-, 2.1-, and 2.1-fold increase of TLR4 expression on thioglycolate-elicited primary macrophages, respectively.

Subsequently, the effect of CRF peptides on TLR4 expression was examined at the transcriptional level. First, real-time RT-PCR was used to evaluate the TLR4 mRNA levels following exposure to CRF peptides at a concentration of 10^{-8} M for 24 h. Exposure of RAW 264.7 cells to CRF, UCN1, and UCN2 increased TLR4 mRNA steady-state levels by 3.2 ± 0.22-, 2.2 ± 0.08-, and 2.5 ± 0.02-fold, respectively, while treatment with LPS reduced TLR4 mRNA levels to 0.75 ± 0.1 of that measured in parallel untreated cells ($n = 3$, mean ± SE, Fig. 2*A*). These results were confirmed using a semiquantitative RT-PCR approach (data not shown). A similar effect was observed using concentrations of 10^{-7} and 10^{-9} M. However, at 10^{-10}M the effect was significantly lower (data not shown). Treatment of mouse primary, thioglycolate-elicited peritoneal macrophages with CRF, UCN1, or UCN2 revealed that all three neuropeptides induce the expression of TLR4 (Fig. 2*B*), confirming the results obtained in RAW 264.7 macrophages. Specifically, following a 24-h incubation period, CRF up-regulated TLR4 mRNA 7.7 ± 0.45-fold, UCN1 6.7 ± 0.8-fold, and UCN2 8.7 ± 0.4-fold, compared with untreated cells ($n = 3$, mean ± SE). Treatment of thioglycolate-elicited peritoneal macrophages with any of the three CRF-related peptides for different time points induced TLR4 mRNA expression, which was first observed at 2 h following the application of the stimulus and was the highest at 24 h (Fig. 2*C*). The results shown are representative of three independent experiments.

To confirm that the effect of CRF, UCN1, and UCN2 on TLR4 expression occurred at a transcriptional level, RAW 264.7 cells were transfected with a construct containing the minimal promoter of mouse TLR4 linked to the luciferase gene. A 550-bp fragment of the mTLR4 promoter was used which is primarily controlled by three proximal PU.1-binding sites (28). Similar results were obtained using a construct containing a fragment between −518 to + 223 bp of the TLR4 promoter (26). Transfected cells were exposed to CRF, UCN1, or UCN2 for 18 h before measuring luciferase activity. Each of CRF neuropeptides tested activated the promoter of TLR4, indicating that their effect occurs at the transcriptional level (Fig. 3*A*). Indeed, CRF, UCN1, and UCN2 increased transcriptional activity of the TLR4 promoter 5.3 ± 0.4-fold, 5.5 ± 0.75-fold, and 5.6 ± 0.25-fold, respectively ($n = 3$, mean ± SE) compared with parallel control (untreated) cells. To define whether the PU.1 and the AP-1-binding sites of the TLR4 promoter were required for CRF peptide action, we first used a construct that included a mutated proximal PU.1 site (PU.1/Ets^{p}) (26). Disruption of the proximal PU.1 site totally abrogated TLR4 promoter responsiveness to CRF peptides (Fig. 3*B*). Similarly, mutation of the AP-1-binding site of the TLR4 promoter abolished the effect of the CRF peptides (Fig. 3*C*), suggesting that the presence of both sites is necessary for CRF receptor signals to drive expression from the TLR4 promoter. The term "NP" denotes luciferase activity of mock DNA-transfected cells while the term "control" denotes luciferase activity of transfected but untreated cells. Each bar corresponds to the average of three individual transfections done in parallel. The depicted data are representative of three independent experiments.

CRF peptides induce nuclear translocation and DNA binding of the transcription factor PU.1

TLR4 expression is primarily controlled by members of the Ets family of transcription factors including PU.1 (28). Because CRF and UCNs had a positive effect on TLR4 promoter activity through

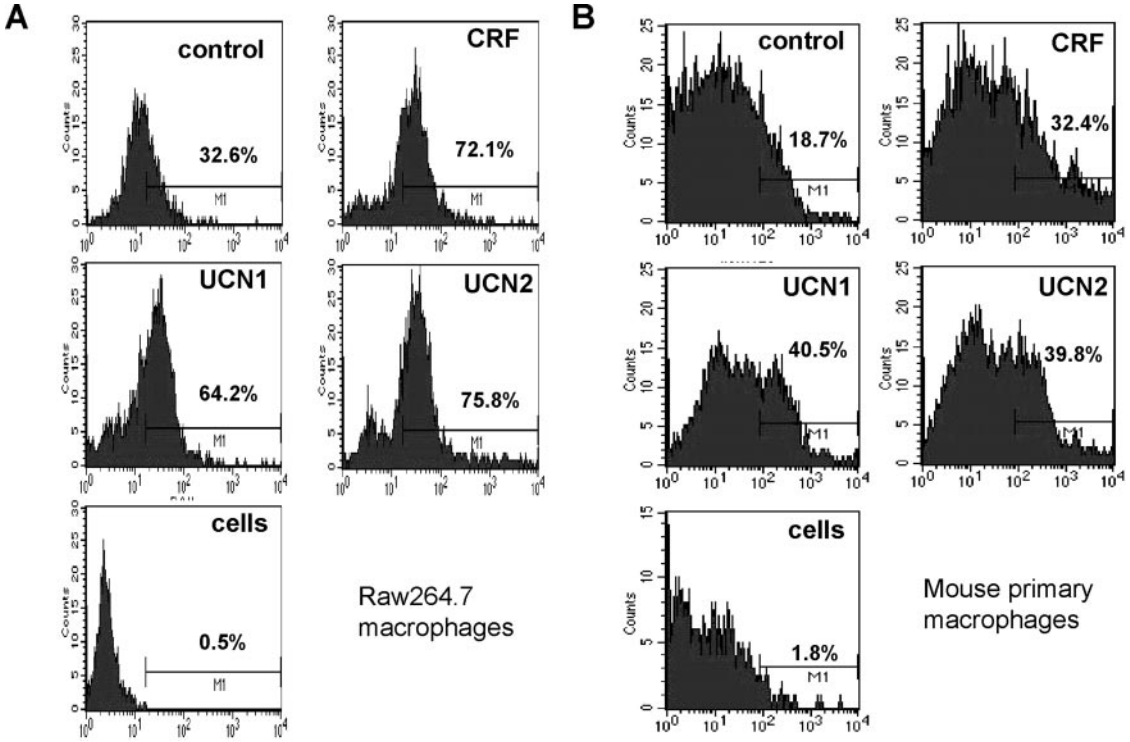

FIGURE 1. Effect of CRF peptides on TLR4 protein. CRF, UCN1, and UCN2 augmented TLR4 protein level both in both RAW 264.7 macrophages and primary thioglycolate-induced peritoneal mouse macrophages as determined by FACS analysis using a TLR4-PE conjugated Ab. *A*, FACS analysis of TLR4 expression by RAW 264.7 macrophages. *B*, FACS analysis of TLR4 expression in thioglycolate-induced peritoneal macrophages. "Control" represents TLR4 staining in untreated cells and "cells" represent background staining with an isotype control Ab. Results are representative of three independent experiments.

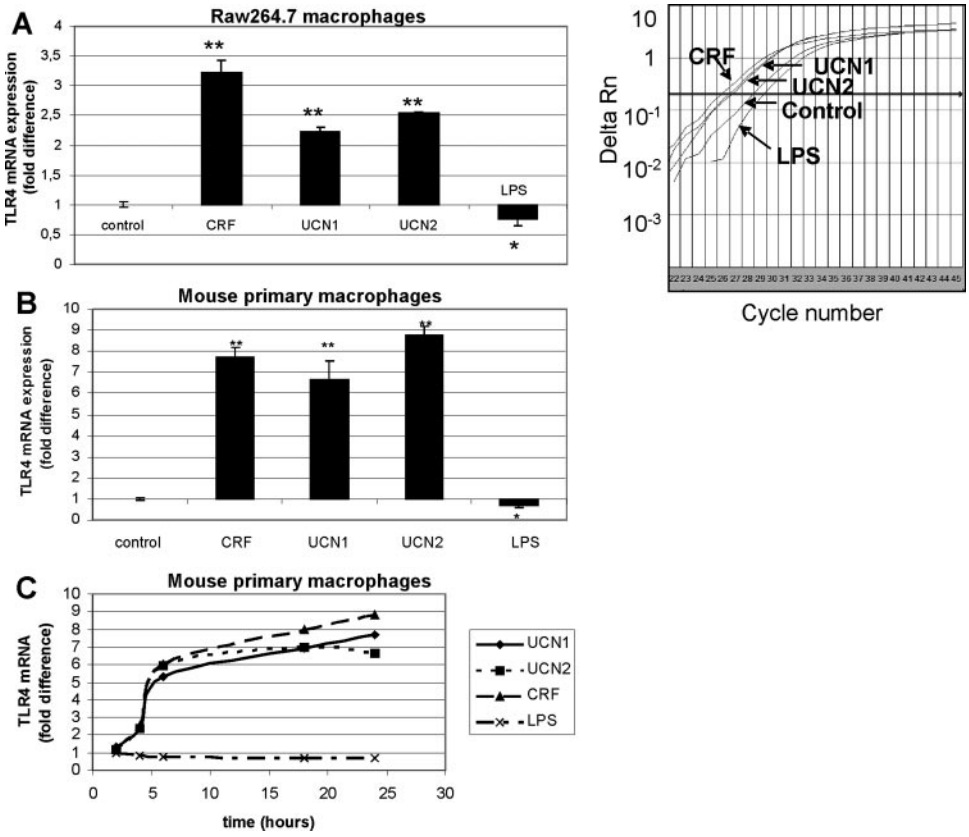

FIGURE 2. Effect of CRF peptides on *Tlr4* gene expression. *A*, Real-time RT-PCR analysis. RAW 264.7 cells were stimulated with CRF, UCN1, UCN2, or LPS and TLR4 expression was measured 24 h following stimulation. Real-time RT-PCR using SYBR Green as DNA dye and ROX as passive dye were used to measure the levels of the TLR4 transcript. The levels of β-actin expression were used for normalization purposes. Counting the ΔCt of each sample and normalizing against the ΔCt of β-actin expression in the same sample, we estimated fold increase. *B*, Mouse primary thioglycolate-elicited peritoneal macrophages were treated with CRF, UCN1, UCN2, or LPS and TLR4 expression was measured by real-time RT-PCR after 24 h. *C*, Mouse primary thioglycolate-elicited peritoneal macrophages were treated for different time periods with the corresponding peptides or LPS. RNA was isolated and expression of TLR4 mRNA was measured by real-time RT-PCR and normalized against the mRNA expression levels of β-actin. Results are representative of three independent experiments. (*, $p < 0.05$ and **, $p < 0.01$ denote statistically significant differences compared with control).

a conserved PU.1 site, we tested whether PU.1 nuclear translocation and DNA-binding activity was affected by CRF and UCNs. RAW 264.7 cells were treated with CRF, UCN1, or UCN2 and translocation of PU.1 into the nucleus was assessed by immunofluorescence analysis, revealing that PU.1 translocated into the nucleus in the presence of each neuropeptide tested (Fig. 4*II* and data not shown). To confirm our observation with a functional assay, we measured PU.1 DNA-binding activity in nuclear lysates of RAW 264.7 macrophages 1 h following stimulation with CRF peptides. Incubation of nuclear protein extracts from CRF, UCN1, or UCN2 treated cells with a radiolabeled oligonucleotide containing the proximal PU.1-binding sequence of the TLR4 promoter resulted in increased DNA-binding activity as shown by EMSA (Fig. 5*A*, *lanes 1–4*). Binding activity was not observed when nuclear extracts were incubated with a radiolabeled mutant proximal PU.1 oligonucleotide (Fig. 5*A*, *lane 5*). The specificity of the PU.1 retarded complex was further demonstrated by showing that it was dose-dependently competed by adding 5- to 100-fold excess of unlabeled proximal PU.1 oligonucleotide (Fig. 5*A*, *lanes 8–10*), but not by a 100-fold excess of unlabeled mutant PU.1 oligonucleotide (Fig. 5*A*, *lane 6*). Finally, the specific retarded complex was supershifted using an anti-PU.1 polyclonal Ab (Fig. 5*A*, *lane 7*). Taken together, these data suggest that CRF peptides increase PU.1 transcription factor binding to the proximal PU.1 DNA-binding site of the TLR4 promoter.

CRF peptides induce binding to the AP-1 site of the TLR4 promoter

An AP-1 site in the proximal region of the TLR4 promoter has also been involved in mediating basal TLR4 promoter activity (26). As reported in Fig. 3*C*, mutation of the AP-1 site of the TLR4 promoter abolished the response to CRF, UCN1, and UCN2. Moreover, previous reports have demonstrated that CRF activates AP-1 binding in keratinocytes and chromaffin cells (29–31). Therefore, we tested whether the AP-1 site mediates the effect of CRF peptides on TLR4 expression. Thus, we treated RAW 264.7 macrophages with CRF, UCN1, or UCN2 for 1 h, prepared nuclear protein extracts, and assessed AP-1-binding activity by EMSA using a labeled TLR4-specific AP-1 oligonucleotide (Fig. 5*B*). All three peptides induced a potent AP-1 binding that was not observed when nuclear extracts were incubated with a labeled mutant AP-1 oligonucleotide (Fig. 5*B*, *lanes 1–5*). Complex formation using the wild-type AP-1 oligonucleotide was specific, because it was dose-dependently inhibited in the presence of an excess of unlabeled AP-1 oligonucleotide (Fig. 5*B*, *lanes 7–9*), while it was not affected in the presence of unlabeled mutant AP-1 oligonucleotide (Fig. 5*B*, *lane 6*).

The induction of TLR4 expression by CRF peptides is mediated by the CRF_2 receptor

CRF and UCN1 signal via both the CRF_1 and CRF_2 receptors, while UCN2 signals exclusively via CRF_2, suggesting that the effect of the neuropeptides may be mediated at least partly by the CRF_2 receptor. To test this hypothesis, we treated RAW 264.7 macrophages with CRF, UCN1, or UCN2 in the presence or absence of the CRF_2 inhibitor anti-sauvagine-30 in a 100-fold excess to ensure complete inhibition (35). Treatment with anti-sauvagine-30 blocked the activation of the TLR4 promoter induced by CRF, UCN1, or UCN2 (Fig. 6*A*). Conversely, the CRF_1 antagonist antalarmin did not appear to have any apparent effect on the activation of the TLR4 promoter by either CRF or UCN (Fig. 6*B*). The potency of our antalarmin compound was also checked in parallel experiments where it inhibited CRF_1 effects as previously reported (11, 32). These findings suggest that all three CRF peptides use CRF_2 to promote transcription from the TLR4 promoter.

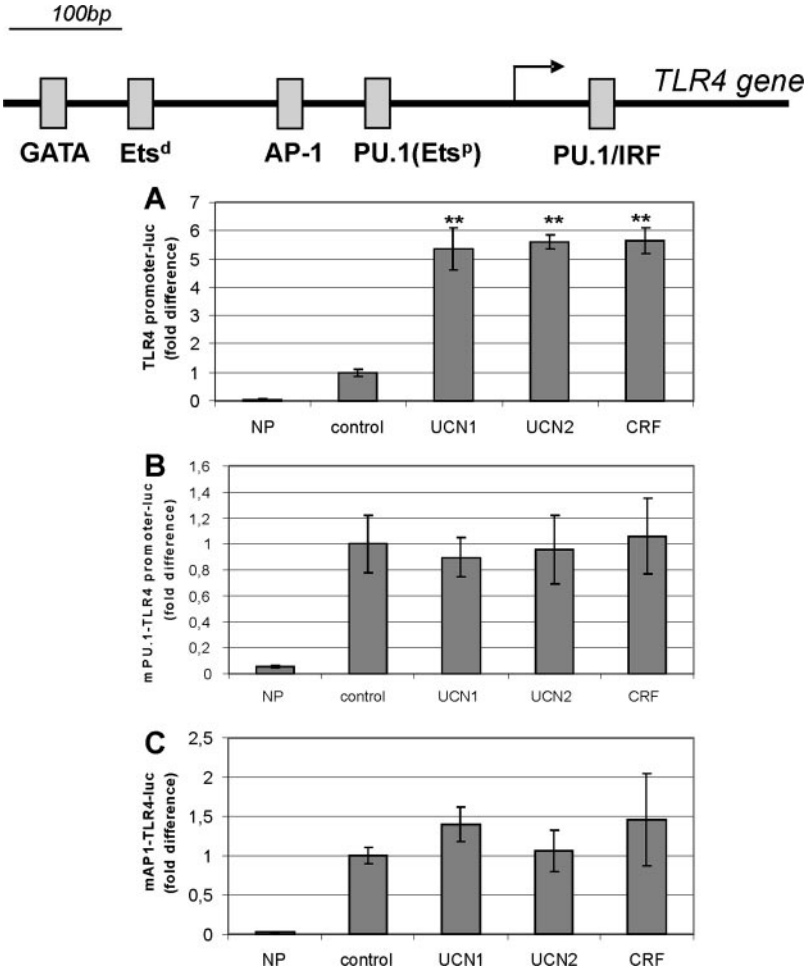

FIGURE 3. Effect of CRF peptides on the TLR4 promoter. UCN1, UCN2, and CRF induced transcriptional activity of the TLR4 promoter. *A*, RAW 264.7 macrophages were transfected with a construct containing the proximal TLR4 promoter linked to the luciferase gene and transcriptional activity was measured 18 h following stimulation with UCN1, UCN2, or CRF. *B* and *C*, RAW 264.7 macrophages were transfected with a construct containing the proximal TLR4 promoter linked to the luciferase gene, including a mutation on the proximal PU.1 site (Ets^p) (*B*) or the AP-1 site (*C*), and transcriptional activity was measured after 18 h of exposure to the corresponding neuropeptide. NP, cells transfected with mock DNA; control, untreated cells. (**, $p < 0.01$ denotes statistically significant differences, compared with control).

CRF peptides prevent LPS-mediated suppression of TLR4 expression

LPS is known to down-regulate mouse TLR4 expression levels (36). Thus, we wanted to determine whether CRF, UCN1, or UCN2 have any impact on the effect of LPS on TLR4 expression. RAW 264.7 cells were transfected with the minimal TLR4 promoter and treated with LPS in the presence or absence of CRF, UCN1, or UCN2 before measuring luciferase activity. Treatment with any of the three CRF peptides partly reversed the negative effect of LPS on TLR4 promoter activation (Fig. 7). Indeed, whereas LPS alone decreased TLR4 promoter activity by 60% (from 16 ± 0.7 in resting cells to 6.2 ± 1.1 in LPS-stimulated cells; $n = 3$, mean luciferase activity ± SE), the presence of CRF peptides ameliorated the suppressing effect of LPS on the TLR4 promoter by reducing its activity by only 26, 37, and 39% for CRF, UCN1, and UCN2, respectively (mean luciferase activity ± SE of 11.8 ± 1.8, 10.1 ± 0.8, and 9.8 ± 0.31). In agreement with our luciferase activity data, real-time RT-PCR suggested that treatment of RAW 264.7 macrophages with CRF, UCN1, or UCN2 attenuated the suppressive effect of LPS on TLR4 mRNA expression (Fig. 8*A*). Specifically, whereas LPS suppressed TLR4 mRNA expression to 0.75 ± 0.10-fold, compared with resting cells, the simultaneous presence of CRF, UCN1, or UCN2 and LPS reversed the negative effect of LPS and up-regulated TLR4 mRNA levels to

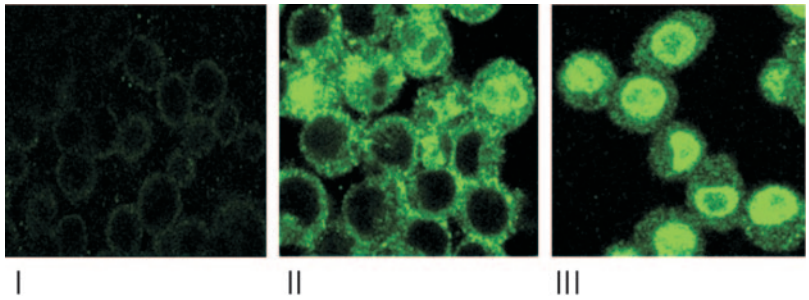

FIGURE 4. CRF peptides induced nuclear translocation of the transcription factor PU.1. RAW 264.7 cells were treated for 1 h with UCN1, UCN2, or CRF. Cells were fixed in 2% paraformaldehyde and PU.1 subcellular localization was detected by immunofluorescence. *I*, Cells stained with secondary Ab only (anti-rabbit-FITC conjugated); *II*, untreated control cells in which PU.1 is visualized primarily in the cytoplasm but is also detected in both the cytoplasm and the nucleus of some cells. *III*, Cells treated with UCN1 for 1 h where PU.1 is visualized in the nucleus of the cells. Similar nuclear translocation patterns were observed in cells treated with UCN2 or CRF (data not shown). Results are representative of three independent experiments.

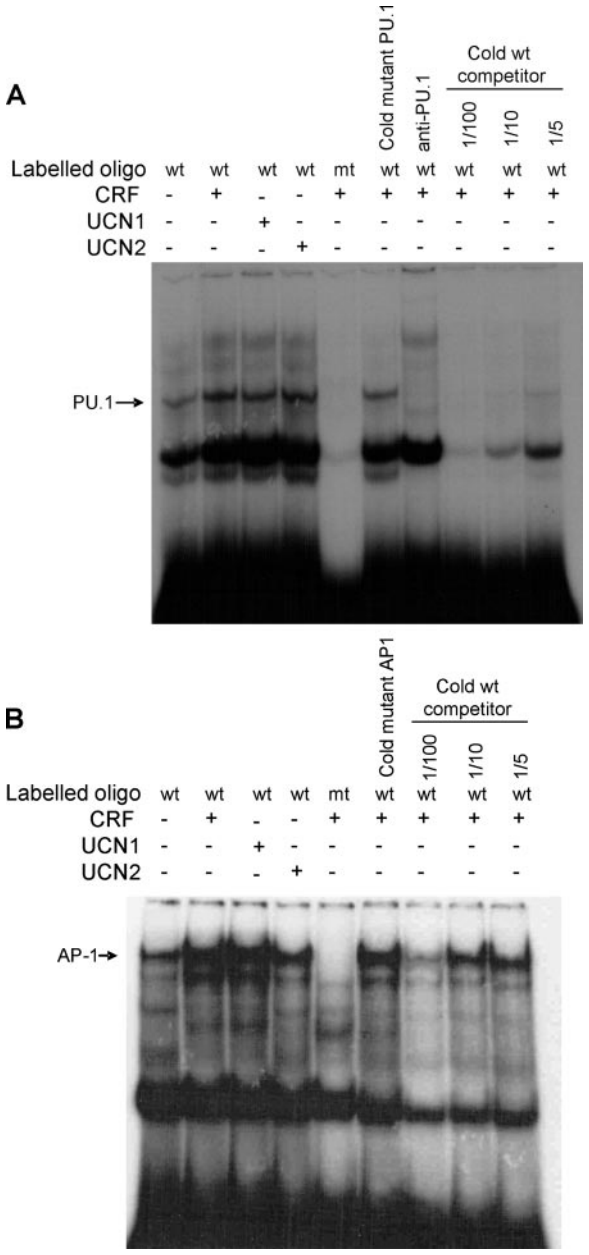

FIGURE 5. CRF and UCNs induced PU.1- and AP-1-binding activity. RAW 264.7 cells were stimulated for 1 h with UCN1, UCN2, or CRF and PU.1 (*A*) or AP-1 (*B*). DNA-binding activity was measured by EMSA. Results are representative of four independent experiments. *Lanes 1–4* represent binding on a labeled wild-type (wt) and *lane 5* on a labeled mutant oligonucleotide (mt). *Lane 6* represents binding on a labeled wild-type oligonucleotide competed with a 100-fold excess of unlabeled mutant oligonucleotide. *Lane 7* in *A* represents binding on the wild-type oligonucleotide in the presence of anti-PU.1 Ab. *Lanes 8–10* in *A* and *7–9* in *B* represent binding on labeled wild-type oligonucleotide in the presence of an excess of unlabeled wild-type oligonucleotide.

2.7 ± 0.05-, 2.25 ± 0.18-, or 2.5 ± 0.04-fold, respectively, compared with untreated cells. Similar results were also obtained using freshly isolated primary thioglycolate-elicited peritoneal macrophages (Fig. 8*B*). Specifically, LPS suppressed TLR4 mRNA levels 0.68 ± 0.07-fold compared with untreated cells, while coexposure to CRF, UCN1, or UCN2 reversed this effect to 5.2 ± 0.92-, 5.15 ± 1.04-, and 6.32 ± 0.88-fold, respectively ($n = 3$, mean ± SE). The data shown are representative of three independent experiments where each point was measured in triplicate.

Discussion

The ad hoc effect of CRF at the site of inflammation on a direct paracrine/autocrine manner is a well-established concept based on a large number of data published over the past 15 years (1–8). CRF and its related peptides reach the inflammation site either through sensory nerve terminals or via production by local epithelium and/or by the inflammatory cells themselves. A well-established target of CRF and UCN is the mast cell (12–17). We have previously shown that CRF also affects monocytes/macrophages by augmenting LPS-induced cytokine production (11). Our present data provide a possible mechanism via which the CRF neuropeptides achieve their ad hoc effect on macrophages. Our data provide a possible cross-talk mechanism between the stress axes and the immune system at a peripheral level. Specifically, our data suggest that CRF and the UCNs induce the expression of TLR4 in macrophages, the receptor via which LPS induces the production of inflammatory cytokines (Fig. 9). Indeed, exposure of freshly prepared primary murine peritoneal macrophages and of the murine macrophage cell line RAW 264.7 to CRF, UCN1, or UCN2 resulted in a time-dependent induction of TLR4 expression indicated by an increase of its protein and mRNA level. Furthermore, transfection of RAW 264.7 cells with the proximal TLR4 promoter linked to the luciferase gene confirmed the hypothesis that the effect of the CRF peptides occurs principally at the transcriptional level. Furthermore, mutation of the proximal PU.1-binding site (Ets^P) or mutation of the AP-1 site rendered the promoter unresponsive to any of the three CRF neuropeptides, indicating that both sites are essential for the effect of CRF receptor signals on TLR4 transcription. Going one step further, we have found that CRF peptides induced the nuclear translocation and DNA binding of the PU.1 transcription factor, the principal regulator of TLR4 transcription, as well as the DNA binding of the transcription factor AP-1 (Fig. 9). In addition, we have also found that CRF peptides reversed the well-known suppressive effect of LPS on *Tlr4* gene expression, indicating that the CRF peptides may affect a negative regulatory mechanism that involves down-regulation of TLR4 expression. Indeed, treatment of macrophages with LPS results in suppression of TLR4 expression first observed 2 h following stimulation (36, 37). Several studies have implicated the effect of LPS on TLR4 expression as a possible mechanism leading to the development of macrophage tolerance toward further LPS stimulation and to the containment of the inflammatory response (36, 37), although macrophage tolerance is also regulated at the level of TLR4 signaling (38). However, CRF peptides do not appear to exert any effect of their own on proinflammatory cytokine production by RAW 264.7 macrophages or by primary mouse macrophages; they most probably only modify macrophage response augmenting an already established inflammation by increasing the sensitivity of macrophages to inflammatory insults (11).

Macrophage sensitivity to LPS is partly regulated at the level of TLR4 expression. Forced overexpression of TLR4 in macrophages results in increased proinflammatory cytokine secretion in response to LPS (23). Increased expression of TLR4 has also been reported in several experimental models of inflammation including intestinal inflammation (39) and respiratory syncytial virus-induced inflammation (40). In the present report, we propose that the stress-related neuropeptides of the CRF family induce TLR4 expression in macrophages and alter their sensitivity to LPS as we have previously described (11).

The levels of TLR4 mRNA expression in macrophages are regulated at the transcriptional as well as at a posttranscriptional level by altering its stability (25, 41). Our data suggest that the effect of CRF peptides most probably occurs at the transcriptional level

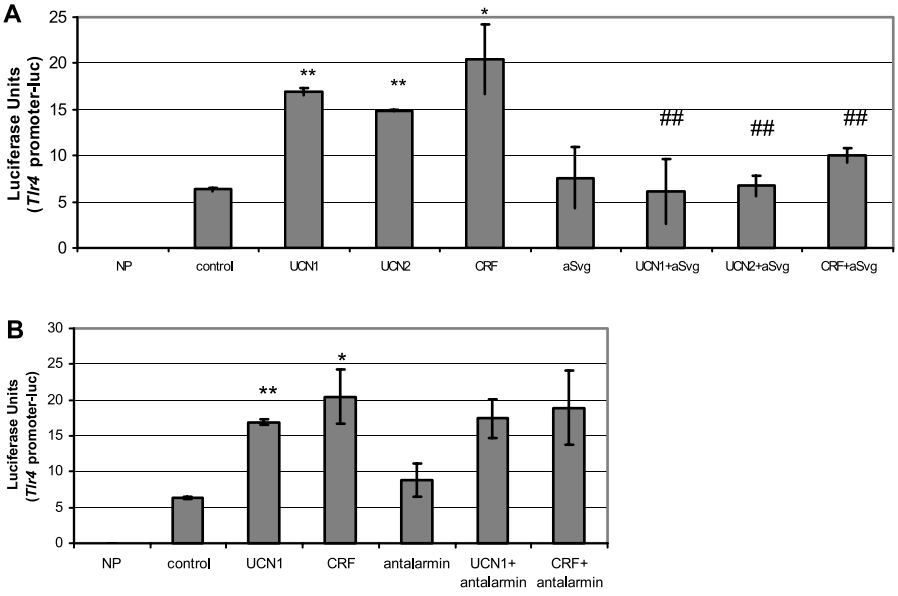

FIGURE 6. Significance of CRF_2 receptor on the effects of CRF peptide-induced TLR4 production. RAW 264.7 cells were transfected with a TLR4 promoter luciferase construct and treated with each of the three neuropeptides in the presence or absence of the CRF_2 antagonist anti-sauvagine-30 (*A*) or the CRF_1 antagonist antalarmin (*B*). (*, $p < 0.05$, and, **, $p < 0.01$ denote statistically significant difference, compared with control, ##, $p < 0.01$, denotes statistically significant difference compared with the corresponding neuropeptide-only treated cells).

because treatment of RAW 264.7 cells with CRF peptides induced transactivation of the minimal promoter of mTLR4. The effects observed in RAW 264.7 cells are not restricted to this particular cell line because primary, thioglycolate-elicited peritoneal macrophages responded in a similar to RAW 264.7 cells manner to CRF, UCN1, or UCN2 stimulation exhibiting an elevation of TLR4 protein and mRNA levels.

The *Tlr4* gene is regulated at the transcriptional level through activation of transcription factors of the Ets family and primarily PU.1 (25), as well as AP-1 (26). The minimal TLR4 promoter contains three PU.1-binding sites (28). PU.1 is expressed in hemopoietic cells and is important for myeloid cell development and maturation and macrophage differentiation (27). PU.1-deficient mice completely lack macrophages and absence of PU.1 impairs myeloid cell differentiation (42, 43). PU.1 is also important for osteoclast differentiation and maturation (43) and is present in microglia where it is significantly up-regulated during ischemic injury (44). It is, therefore, an important factor for all cells of the macrophage lineage regulating their maturation and activation. PU.1 is reported to participate in neuroimmunomodulation because it induces μ-opiod receptor expression in macrophages (45). In the present study, we present experimental evidence suggesting that CRF induces translocation of PU.1 into the nucleus as well as its binding to DNA.

CRF has been reported to activate the AP-1 transcriptional complex in keratinocytes and the AtT-20 corticotrope-derived cell line (29, 31). The composition of AP-1 triggered by CRF appears to be a complex phenomenon consisting of alternation of c-Fos, JunB, Fra2, and JunD proteins (30). In the present report, we propose that CRF and its homologous peptides UCN1 and UCN2 provoke the binding of AP-1 on the mTLR4 promoter. Interestingly, from what we know, this appears to be the first report suggesting that UCNs activate the AP-1 complex. The composition of the complex upon stimulation of each neuropeptide and at different time points in macrophages is under investigation.

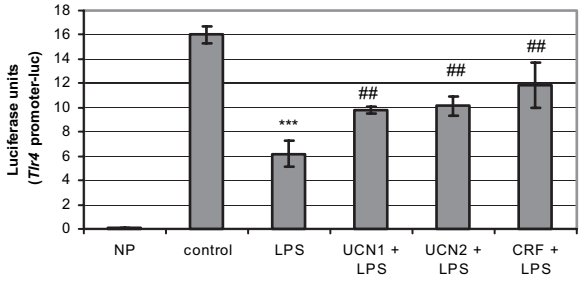

FIGURE 7. CRF peptides prevented the suppressing effect of LPS on TLR4 promoter activation. RAW 264.7 cells were transfected with the TLR4 promoter luciferase construct and treated with UCN1, UCN2, or CRF in the presence or absence of LPS. Luciferase activity was measured 18 h later. "NP" represents cells transfected with mock DNA and "control" untreated cells. (***, $p < 0.001$ denotes statistically significant difference, compared with control, and ##, $p < 0.01$ denotes statistically significant difference, compared with LPS-only treated cells).

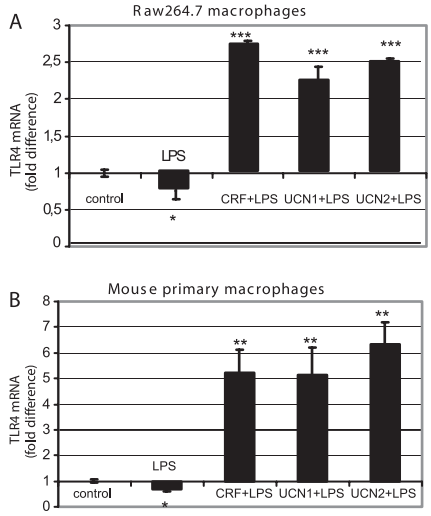

FIGURE 8. CRF peptides prevented the suppressing effect of LPS on TLR4 expression. *A*, RAW 264.7 cells were stimulated LPS in the presence or absence of CRF, UCN1, or UCN2 for 24 h and the expression of TLR4 mRNA was measured by real-time RT-PCR and normalized against the expression of β-actin. *B*, Mouse primary thioglycolate-elicited peritoneal macrophages were stimulated with LPS in the presence or absence of each neuropeptide for 24 h, and expression of TLR4 was measured by real-time RT-PCR. (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$, denote statistically significant difference, compared with LPS-treated cells).

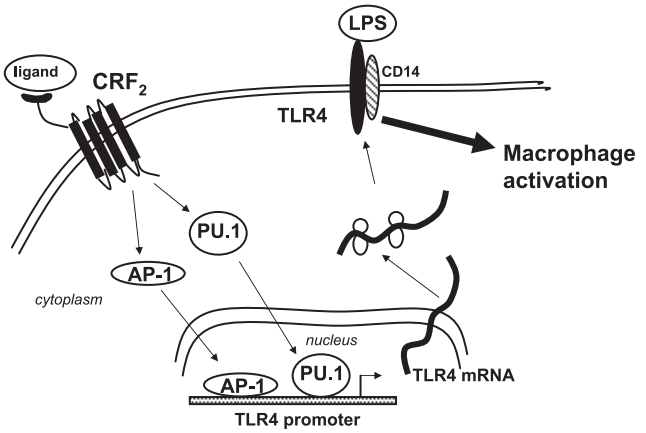

FIGURE 9. Proposed model for the proinflammatory action of CRF_2 signals in macrophages.

Little is known about the exact role of each of the CRF receptors on the immune system. It has been recently shown that the effect of CRF on mast cells is mediated exclusively by the CRF_1 (18), while treatment of human PBMCs with UCN1, a CRF_1, and a CRF_2 agonist induces IL-6 secretion (7). Our data suggest that the effect of CRF and UCNs on the expression of TLR4 involves the CRF_2 receptors toward which CRF exerts a much weaker effect compared with that of its endogenous ligands, the UCNs (46). Macrophages and Kupffer cells appear to possess functional CRF_1 and CRF_2 receptors (Ref. 22 and our unpublished data). Indeed, the CRF_2 antagonist anti-sauvagine-30 inhibited the stimulatory effect of CRF and UCNs on TLR4 expression, while the CRF_1 antagonist antalarmin did not, indicating that this effect is a truly CRF_2 receptor-mediated effect.

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Disclosures

The authors have no financial conflict of interest.

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