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Chronic Morphine-Induced MicroRNA-124 Promotes Microglial Immunosuppression by Modulating P65 and TRAF6

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Opioids have been widely applied in clinics as one of the most potent pain relievers for centuries. However, the abuse of opioids has deleterious physiological effects including immunosuppression. However, the mechanisms are unclear. TLRs and acetylcholine are widely expressed in the immune and nervous systems, and play critical roles in immune responses. In this article, we show that morphine suppresses the innate immunity in microglia and bone marrow–derived macrophages through differential regulation of TLRs and acetylcholinesterase. Either morphine or inhibition of acetylcholine significantly promotes upregulation of microRNA-124 (miR-124) in microglia, bone marrow–derived macrophages, and the mouse brain, where miR-124 mediates morphine inhibition of the innate immunity by directly targeting a subunit of NF-κB p65 and TNFR-associated factor 6 (TRAF6). Furthermore, transcription factors AP-1 and CREB inhibited miR-124, whereas p65 bound directly to promoters of miR-124, thereby enhancing miR-124 transcription. Moreover, acute morphine treatment transiently upregulated the expression of p65 and phospho-p65 in both nucleus and cytoplasm priming the expression of miR-124, whereas long exposure of morphine maintained miR-124 expression, which inhibited p65- and TRAF6-dependent TLR signaling. These data suggest that modulation of miRs is capable of preventing opioid-induced damage to microglia. 

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Abstract

MicroRNAs (miRs) are small and noncoding RNAs that are involved in modulating gene expression at the post-transcriptional level. Their role in immune responses is now well-established. In this study, we investigated the role of miR-124 in morphine-induced microglial immunosuppression. We found that morphine upregulated the expression of miR-124 in microglia and bone marrow–derived macrophages, and the mouse brain, where miR-124 mediates morphine inhibition of the innate immunity by directly targeting a subunit of NF-κB p65 and TNFR-associated factor 6 (TRAF6). Furthermore, transcription factors AP-1 and CREB inhibited miR-124, whereas p65 bound directly to promoters of miR-124, thereby enhancing miR-124 transcription. Moreover, acute morphine treatment transiently upregulated the expression of p65 and phospho-p65 in both nucleus and cytoplasm, priming the expression of miR-124, whereas long exposure of morphine maintained miR-124 expression, which inhibited p65- and TRAF6-dependent TLR signaling. These data suggest that modulation of miRs is capable of preventing opioid-induced damage to microglia.
morphine. In this article, we reveal that morphine represses the innate immune function through upregulation of miR-124. Furthermore, acute morphine treatment transiently upregulated the expression of p65 and phospho-p65 in both nucleus and cytoplasm priming the expression of miR-124, whereas long exposure of morphine maintains miR-124 expression, which inhibits p65- and TRAF6-dependent TLR signaling. These findings suggest that modulation of miRs is capable of preventing opioid-induced damage to microglia.

Materials and Methods

Cell lines and cell cultures

HEK 293T cell line was from American Type Culture Collection. Mouse BV2 microglial cell line was kindly provided by Dr. Gary Landreth (Case Western Reserve University School of Medicine). All cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS in a humidified atmosphere containing 5% CO2 at 37˚C without antibiotics.

Primary microglial cells culture

Mouse primary microglial (PM) cells were isolated from mixed glial cultures as described in our previous studies (5). In brief, primary mixed glial cells were from postnatal day 1–2 BALB/c mice. PM cells were cocultured with astrocytes in DMEM/F12, HEPES medium supplemented with 10% FBS and 1% penicillin/streptomycin (all from Invitrogen). After 24 h, the growth medium was changed with fresh medium. On days 12–14, PM cells were harvested by shaking the cultures (180 rpm, 40–60 min) and collecting the floating cells. The cells were incubated at 37˚C and 5% CO2 for 1–2 d to achieve microglia in a quiescent state. PM cells from the first and the second passage were used (>95% purity).

Bone marrow–derived macrophages culture

Bone marrow–derived macrophages (BMMs) were isolated and cultured as follows (26). In brief, bone marrow was isolated from long bones of 4- to 6-wk-old BALB/c mice and incubated with M-CSF (10 ng/ml; Sigma-Aldrich) in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin for 3 d. The medium was changed every 2 or 3 d. One week later, macrophages were differentiated for assays. BMM cells were used from the first and second passage.

Morphine and TLR agonists treatment on PM and BMM cells

PM and BMM cells were incubated with TLR2 agonist Pam3csk4, TLR4 agonist LPS, TLR5 agonist flagellin, or TLR8 agonist Resiquimod at 10 μM for 2 h and then treated with morphine at 10 μM for another 22 h.

Animals and chronic morphine administration in mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the Division of Laboratory Animal Resources at East Tennessee State University, a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All animal studies were approved by the East Tennessee State University Committee on Animal Care. We followed an established procedure to induce morphine abuse described by us and others (27–30). In brief, 8- to 10-wk-old C57BL/6 male mice received twice-daily injections of an escalating dose of morphine sulfate (Sigma-Aldrich, St. Louis, MO) over 8 d (10, 20, 40, 80, 100, 120, 140, 140 mg/kg i.p.). Age-matched control male mice were treated twice daily with PBS. Twenty-four hours after the last injection, the prefrontal cortex and hippocampus were harvested and kept at −80˚C.

Transfection and injection of miR-124 mimics, miR-124 inhibitor, and small interfering RNA

BV2 cells were transfected with miR-503/124 mimics, negative control (NC) mimics, and miR-124 inhibitors (Ambion, Carlsbad, CA) with the final concentration of 10 nM using the transfection reagent HiPerfect (Qiagen, Germantown, MD). BV2 cells were transfected with siRNA-p65 and siRNACREB (Santa Cruz Biotechnology, Santa Cruz, CA) with the working concentration of 100 nM using Lipofectamine 2000 (Invitrogen). Both miR-124 mimics and inhibitor (Ambion) were complexed with Invitrofectamine 2.0 (Invitrogen) reagent and injected through the tail vein of male C57BL/6 mice on 3 consecutive days at the dose of 7 mg/kg. The mice were treated with miR-124 inhibitor on the last 3 days after morphine treatment. At 24 h after the last injection, the prefrontal cortex and hippocampus were harvested.

Construction and transfection of plasmids

The wild type (wt) 3’-untranslated region (3’-UTR) plasmids of p65 and TRAF6 were cloned using primers (Supplemental Table 1) and inserted into the 3’-UTR of the Renilla luciferase gene of the psiCHECK2 vector (Promega, Madison, WI). The mutant type (mut) 3’-UTR plasmids of p65 and TRAF6 were produced by use of site-directed mutagenesis, using primers (see Supplemental Table I for all primers used in the study). There were three binding sites between miR-124 and TRAF6 3’-UTR, whereas the mut1 plasmid was mutant at binding site 1, mut2 at sites 1 and 2, and mut3 at all three binding sites. The putative promoters of miR-124-1 and miR-124-3 on genome loci were cloned using primers in Supplemental Table I and cloned into upstream of luciferase gene in the pGL3-Basic vector (Promega), namely, pGL3-124-1 and pGL3-124-3. The authenticity of DNA sequences was confirmed by sequencing. BV2 or HEK293T cells were transfected with these plasmids (including p65, p50, and c-fos overexpression plasmids [Addgene, Cambridge, MA]) using Lipofectamine 2000 at 0.5–1.5 μg/ml. Cotransfections for miR-124 mimics and p65 overexpression plasmids were performed by using Lipofectamine 2000.

Western blot

Total proteins were extracted using radioimmuno precipitation assay lysis buffer (Pierce, Rockford, IL). Nuclear and cytoplasmic proteins were extracted separately using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). Protein lysates were separated by 8% SDS-PAGE and then electrophoretically transferred to polyvinyl difluoride membrane (Millipore). The membrane was incubated with either rabbit anti-mouse p65, phospho-p65, p105, and phospho-p105 (1:1000; Cell Signaling Technology, Beverly, MA), mouse anti-mouse TRAF6 (1:2000; Abcam, Cambridge, MA), rabbit anti-mouse lamin B1 (1:2000; Cell Signaling Technology), or rabbit anti-mouse GADPH Ab (1:2000; Cell Signaling Technology) followed by HRP-labeled anti-mouse or goat anti-rabbit IgG (1:5000; Cell Signaling Technology). Protein levels were detected using enhanced chemiluminescence detection solution (Pierce) and visualized on a x-ray film (Kodak).

ELISA for cytokines

The levels of proinflammatory cytokines TNF-α, IL-1β, and IL-6 were analyzed using mouse Quantikine ELISA immunoassay kits (R&D systems, Minneapolis, MN) according to the manufacturer’s instructions.

RNA extraction and evaluation of miR levels

RNA was isolated using the mirVana kit or Qiagen RNeasy kit (Qiagen). Samples after lysis were incubated with DNase digestion kit (Qiagen) to degrade genome DNA. RNA (10 ng) was converted into cDNA with TaqMan MicroRNA Assay and TaqMan Reverse Transcription kit (Applied Biosystems, Foster, CA). Real-time PCRs were performed with specific primers (TaqMan MicroRNA Assay) and TaqMan Universal PCR Master Mix (Applied Biosystems) on Bio-Rad PCR instrument. PCR conditions were performed according to the standard protocol with the 50˚C preincubation for 2 min and 95˚C incubation for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. U6 small nuclear ribonucleoprotein was used as an endogenous control.

Evaluation of mRNA levels

Total RNA was reverse-transcribed using reverse transcription system (Promega). Real-time PCR was performed using SYBR GreenER (Invitrogen) on the Bio-Rad PCR instrument. PCR was performed according to the standard protocol with the 50˚C preincubation for 2 min and 95˚C incubation for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. GAPDH was included as an endogenous control. All real-time PCRs were performed in triplicate, and relative quantifications were calculated using the relative fold changes of cycle threshold method (95% CI). All primer sets were subjected to a dissociation curve analysis and produced single peaks on a derivative plot of raw fluorescence.

Dual-luciferase reporter assay

Validation of miR-124 binding to 3’-UTR was performed using dual-luciferase reporter assays. miRs and p65/TRAF6 wt/mut 3’-UTR plasmids were cotransfected in HEK293T cells for 48 h; then luciferase assays were performed on the Modulus microplate (Turner Biosystem) using the dual-luciferase reporter assay system (Promega). These experiments were repeated at least three times in triplicates. The relative luciferase activities were determined by calculating the ratio of Renilla luciferase activities over firefly luciferase activities. Validation of the transcriptional factor p65 binding to putative promoters of miR-124 was carried out with the dual-
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Signaling participating in immune response in our model, and suggest that morphine inhibits the innate immunity in microglia, we found that chronic morphine treatment suppressed the expression of TNF-α, IL-1β, and IL-6, which was increased by overexpression of miR-124 inhibitors (Fig. 2G). Like in BV2 cells, these proinflammatory cytokines were decreased in PM cells (Fig. 2H). To define whether miR-124 participated in morphine-elicited downregulation of TNF-α, BV2 and PM cells were treated with miR-124 inhibitors and morphine. We found that morphine counteracted miR-124 inhibitor-induced TNF-α production in BV2 (Fig. 2I) and the expression of IL-1β in PM cells (Fig. 2J). Collectively, our results suggest that morphine-induced upregulation of miR-124 inhibits the production of proinflammatory cytokines.

We then assessed the effect of ACh on miR expression using ACh receptor antagonist atropine. We showed that either short- or long-time atropine treatment did not alter the expression of miR-124 or miR-132 in PM cells, but short-time atropine promoted upregulation of miR-124 in PM cells and long-time atropine significantly downregulated miR-132 in BMM cells (Supplemental Fig. 2B, 2C). Meanwhile, we found that expression of miRs was altered both in PM and BMMs treated with no FBS. Serum withdrawal increased miR-124 expression but decreased the expression of miR-132 in BMMs (Supplemental Fig. 2B, 2C). Taken together, these data suggest that cholinergic signaling plays a role in modulation of miR-124 and miR-132 in immune response.

miR-124 suppresses microglial immune function by targeting p65 and TRAF6

miRs function by inhibiting the expression level of targeting genes. To search for potential targets of miR-124 with TargetScanMouse algorithm, we found that p65 and TRAF6 are potential targets of miR-124 (Fig. 3A). To verify whether p65 and TRAF6 are indeed targets of miR-124, we determined luciferase activity by dual-luciferase reporter assay in HEK293T cells transfected with wt and mut 3′-UTR plasmids. We showed that miR-124 reduced the luciferase activity of p65 wt-UTR by ~40% in comparison with that of NC, whereas miR-124 did not alter the luciferase activity of p65 mut UTR, indicating that miR-124 directly bound to the putative binding site of p65 3′-UTR (Fig. 3B). Compared with NC, miR-124 decreased almost 50% of luciferase activity of TRAF6 wt-UTR, suggesting a direct interaction between miR-124 and 3′-UTR of TRAF6. Interestingly, both luciferase activities of TRAF6 mut1-UTR and mut2-UTR were restored to ~70%, suggesting miR-124 bound to site 1 rather than site 2 (Fig. 3B). Meanwhile, luciferase activities of TRAF6 mut3-UTR were re-
Morphine inhibits p65 and TRAF6 through miR-124

We next determined the molecular mechanism by which morphine modulates p65 and TRAF6. We showed that morphine reduced the expression of p65 and TRAF6 in a dose-dependent manner (Fig. 4A) and downregulated TRAF6 level in a time-dependent manner (Fig. 4B). Moreover, morphine decreased levels of both p65 and TRAF6 in PM cells (Fig. 4C). LPS significantly activated translation of p65 in BV2 cells were also significantly reduced with various time periods (Fig. 5B, 5C). Conversely, with acute administration of morphine, transcription of p65 increased and peaked at 3 h in BMM cells, which corresponded with the changes of IL-6 and TRAF6 (Fig. 5B, Supplemental Fig. 2D). Similarly, expression of phospho-p65 and p65 in nucleus and cytoplasm increased after short-time morphine treatment (Fig. 5C). The expression of p65 both in nucleus and in cytoplasm increased after short-time morphine treatment (Fig. 5C). Thus, these data suggest that all p65, AP-1, and CREB are involved in morphine-mediated biological processes in microglia and that morphine dynamically modulates expression of the transcriptional factor p65.

Transcription factor p65 promotes miR-124 transcription

We next determined the regulation of p65, AP-1, or CREB on miR-124. Overexpression of c-Fos in BV2 cells significantly inhibited expression of miR-124 (Fig. 6A), whereas inhibition of CREB dramatically induced miR-124 expression (Fig. 6B). However, overexpression of p65 and p50 significantly upregulated the expression of mature miR-124 (Fig. 6C) and transcription of both pri-miR-124-1 and pri-miR-124-3 other than pri-miR-124-2 (Fig. 6D). Moreover, knockdown of p65 led to a significant reduction of miR-124 by ~80% (Fig. 6E). These data strongly suggest that both AP-1 and CREB negatively, whereas p65 positively, regulate transcription of miR-124.
To define how p65 promoted miR-124 transcription, we analyzed via the UCSC Web site (http://genome.ucsc.edu/) the genomic loci that may be affected by miR-124. As shown in Fig. 7A, miR-124-1, miR-124-2, and miR-124-3 are located within three different chromosomes, chr14, chr3, and chr2, respectively. Using the computer program MAPPER, a search engine to identify TF binding sites (32), we examined the ~1.8-kb DNA sequence upstream of the AK044422 start codon (miR-124-1), ~2.0-kb DNA sequence upstream of AK011787 start codon (miR-124-2), and ~2.1-kb DNA sequence upstream of precursor miR-124-3. We observed that there are two binding sites for each miR-124 transcript (Fig. 7B, 7C). To verify whether these two binding sites are authentic and active, we cloned the DNA sequences incorporating the binding sites and inserted them upstream of luciferase genes of pGL3 basic vectors. The luciferase reporter assay showed that luciferase activities were increased nearly 6-fold in comparison with empty vector (Fig. 7D). Taken together, our results reveal that the transcription factor p65 promotes miR-124 transcription.

**Discussion**

Opioid abuse such as chronic morphine administration diminishes immune functions; however, the molecular mechanisms by which opioids affect immune cell functions remain to be elucidated. In this study, we find that chronic administration of morphine damaged the innate immunity and promoted expression of miR-124 in microglia cells and mouse brain tissues. Morphine-induced immune suppression is mediated through upregulation of miR-124, which directly inhibits the TLR downstream p65 and TRAF6. Moreover, acute morphine activated, whereas chronic morphine significantly deactivated, expression of both phospho-p65 and p65, dynamically modulating transcription of miR-124. Morphine may initially prime the expression of miR-124 because of the activation of p65 and later maintain inhibition of TLR signaling through miR-124–dependent inhibition of p65. Our findings therefore provide a novel mechanism for understanding the potential mechanism associated with the immunosuppressive effect of opioids abuse.

Recent studies have shown that miRs play an important role in regulation of genes of the immune system (18), including in macrophages, microglia, dendritic cells, and T cells (19). miRs participate in immune responses through modulation of TLR signaling. First, miRs directly regulate TLRs themselves at regulatory sites (33). miRs can also participate in immune responses through modulation of TLR signaling. First, miRs directly regulate TLRs themselves at regulatory sites (33). miRs may also activate immune responses through modulation of TLR signaling. First, miRs directly regulate TLRs themselves at regulatory sites (33). miRs may also activate immune responses through modulation of TLR signaling. First, miRs directly regulate TLRs themselves at regulatory sites (33).
cell levels. It has been confirmed that miR-223 is a regulator of NF-κB pathway post bacterial infection (42, 43). These studies reveal that miRs are important regulators which is regulated by miRs as well to modulate immune responses.

Intriguingly, in our study, alteration of TLR expression by morphine in microglia varied from majority increased to minority decreased. Moreover, morphine inhibited IL-1β and IL-6 production induced by TLR2 agonist and LPS, whereas synergistically promoting those induced by TLR5 and TLR8 agonists. These suggest different roles of TLRs in morphine induction of immune function in microglia and also that the integrated inhibitory function of morphine on immunity is attributed to suppression of TLR downstream molecules TRAF6 and p65, rather than TLRs themselves. This provides a new explanation for morphine-mediated immune suppression. It is well-known that neuroendocrine reflex takes part in inflammation, with AChE-ACh signaling involved in this process. Soreq’s group (8) demonstrated that upregulation of miR-132 potentiates ACh-mediated anti-inflammation through targeting AChE in LPS-induced inflammation model in vitro and in vivo. In support of this study, downregulation of miR-132 varied inversely with AChE in morphine-induced inflammation model of macrophages. Compared with the LPS model, however, this phenomenon was not observed in the microglia model (Supplemental Fig. 2A), which is consistent with the finding that atropine did not induce significant miR-124 expression and that miR-124 did not target AChE in PM cells (Supplemental Fig. 2B, 2C, 2E). This could be because of the slight and cumulative suppression of immunity by morphine. Although upregulation of miR-124 is not by serum

![FIGURE 3.](http://www.jimmunol.org/) p65 and TRAF6 are direct targets of miR-124. (A) Putative binding sites between miR-124 and 3′-UTRs of p65 or TRAF6 were predicted by TargetScanMouse. All the wt and mut 3′-UTR fragments were cloned into psiCHECK2 vectors. The mutant 3′-UTR vectors were produced by the approach of site mutations. TRAF6 mut 1 vectors were mutated at binding site 1, mut 2 vector at binding sites 1 and 2, and mut 3 vector at all three binding sites. (B) Confirmation of miR-124 binding to 3′-UTRs of p65 or TRAF6 was performed by dual-luciferase activity reporter assay. Empty psiCHECK 2 vector was a control of UTR vectors, and NC oligonucleotides were controls of mimic. ***p < 0.001 versus NC. (C) BV2 Cells were transfected with either miR-124 mimics or inhibitors at 10 nM using HiPerfect reagent. NC oligonucleotides were as controls. Expression of p65 and TRAF6 proteins was assessed by immunoblot. ***p < 0.001. (D) PM cells were transfected with miR-124 mimics or NC at 10 nM using HiPerfect reagent. The expression of p65 and TRAF6 was determined by real-time PCR. (E) Eight- to 10-wk-old C57BL/6 male mice (n = 5/group) were treated with in vivo ready miR-124 mimics or NC through tail-vein injection for 3 consecutive days (7 mg/kg body weight). Twelve hours after the last injection, the prefrontal cortex and hippocampus were collected and the expression of p65 and TRAF6 were determined by immunoblot.
treatment, we speculated that this could be attributed to starvation-induced effect instead of AChE, which needs further confirmation. In this study, our results suggest that AChE exerted less function in morphine-induced inflammation in microglia than that in macrophage.

In this study, we found that long-term morphine treatment inhibits production of proinflammatory cytokines and the expression of microglial activation marker CD11b. This is supported by the immunoblot result that morphine downregulated proteins TRAF6 and p65 both in vitro and in vivo, suggesting that long-term morphine exposure attenuates the immune function of microglia in the CNS. This is consistent with Franchi's (9) report that morphine exposure downregulated the expression of TLR4 mRNA and protein in RAW264.7 and peritoneal macrophages. Another recent study also found that acute and chronic morphine evoke a differential expression of cytokines in the rodent brain, with chronic

FIGURE 4. Morphine inhibitory effect on p65 and TRAF6 through miR-124. (A) BV2 cells were incubated with various doses of morphine (MOR) for 24 h, and the expression of p65 and TRAF6 were examined by immunoblot. (B) BV2 cells were treated with different times of MOR at 10 μM, and TRAF6 expression was determined as in (A). (C) PM cells were added with MOR at 10 μM for 24 h, and the expression of p65 and TRAF6 were determined by real-time PCR. **p < 0.01. (D) PM cells were incubated with LPS at 10 μM for 2 h and then treated with MOR at 10 μM for 22 h. The expression of p65 and TRAF6 were assessed as in (C). ***p < 0.01 versus PBS, *p < 0.01 versus LPS. (E) BV2 cells were transfected with miR-124 inhibitors (INHs) for 24 h and then treated with MOR at 10 μM for 24 h. The expression of p65 and TRAF6 were determined as in (A) and subsequent analysis of gray values (F). *p < 0.05, **p < 0.01 versus NC + PBS, #p < 0.01 versus INH + PBS. (G) Eight- to 10-wk-old C57BL/6 male mice (n = 5/group) were i.p. injected with MOR or PBS for 8 consecutive days. On days 6, 7, and 8, mice were also injected with miR-124 INHs or NC through tail veins. Twelve hours after the last morphine injection, brain tissues were collected for immunoblot.

FIGURE 5. Morphine (MOR) dynamically modulates the expression of p65 in microglia. (A) BV2 cells were incubated with MOR at 10 μM for different times, and the expression of c-FOS, p-c-FOS, c-JUN, p-CREB, and CREB were examined by immunoblot. (B) PM and BMM cells were incubated with MOR at 10 μM for different times, and expression of p65 was examined by real-time PCR. (C) BV2 cells were treated with MOR at 10 μM for different times, and proteins in nucleus and cytosol were extracted separately. Expression levels of phospho-p65, p65, phospho-p105, and p105 in nucleus and cytosol were evaluated as in (A). Lamin B was the endogenous control, whereas GAPDH was the exogenous control.
MORPHINE-INDUCED miR-124 PROMOTES IMMUNOSUPPRESSION

morphine significantly lowering expression of IL-1β (46). However, studies from Hutchinson’s group (47–50) showed the opposite result where morphine increased proinflammatory cytokines in macrophages and promoted activation of microglia in spinal cord, and these effects are probably mediated through TLR4. Fukagawa et al. (15) reported that microglia activation induced by morphine is independent of TLR4 in spinal cord using the TLR4 mutation and deletion mice, although CD11b expression was significantly increased after morphine treatment. Despite opioids such as morphine playing important roles in immunosuppression, it is unclear why discrepancies occur among these studies. We speculate that the different concentrations and various treatment periods with morphine are important causes for these differences. For example, the concentration of morphine, 100 μM for cell treatment, in Hutchinson’s study is high enough to induce toxicity rather than physiological function, resulting in cell apoptosis (14), because in their studies, concentrations <100 μM seem not to elicit significant changes in IL-1β and TLR4 mRNAs. Moreover, the different types of morphine among these studies may also contribute to these discrepancies. However, a similar effect of morphine on TLR-mediated signaling through classic μ-opioid receptor was observed in our studies and in Franchi’s report (5, 9).

The regulation of p65 by morphine remains to be defined. Some investigations reported that morphine is able to increase p65 activity in various cell types (51–53), whereas others reported the opposite effect (54). Consistent with Börner et al.’s report (55) in neuronal cells, our study showed that morphine exposure tran-

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** AP-1 and CREB negatively, but p65 positively, regulate transcription of miR-124. BV2 cells were transfected with c-FOS vector (A), siRNA CREB (B), or p65/p50 vectors (C) for 48 h, and miR-124 expression was determined by real-time PCR. The corresponding empty vector was an NC. (D) BV2 cells were transfected with p65 or p50 vectors as in (C). The genome was first removed before mRNAs were converted into cDNA. Then quantification of three pri-miR-124s was determined as in (A). (E) BV2 cells were transfected with siRNA p65 for 48 h and miR-124 expression was assessed as in (A). **p < 0.01, ***p < 0.001.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** The transcription factor p65 promotes transcription of miR-124. (A) miR-124-1 was located within the fourth exon of the gene AK044422 on chromosome (Chr) 14. There were two p65 binding sites within the ~1.8-kb DNA sequence upstream of the AK044422 start codon, and this sequence was cloned into pGL3 basic vector and designated as pGL3-124-1. miR-124-2 was located between exons 2 and 3 of the gene AK011787 on Chr 3. miR-124-3 was harbored on Chr 2, and the ~2.1-kb DNA sequence upstream of precursor miR-124-3 was predicted as promoter region. There were two binding sites for p65 in this fragment, and it was cloned into pGL3 basic vector and designated as pGL3-124-3. (B) BV2 cells were transfected with p65 overexpression and empty vectors for 48 h before the ChIP assay was performed. The chromatin from BV2 cells was immunoprecipitated by using Abs against p65 protein. Shown are the binding sites of p65 to pGL3-124-1 and pGL3-124-3 detected by real-time PCR analysis. Ab against IgG was included as an NC. (C) Gel graph of real-time PCR result after ChIP assay for putative binding sites P1 and P3 from (B). (D) HEK 293T cells were cotransfected with miR-124 promoter vectors (pGL3-124-1 and pGL3-124-3), p65 overexpression vectors, and pRL-TK Renilla vectors (empty vector) for 48 h, and luciferase activities were measured using the dual-luciferase reporter assay system.
siently increased and then decreased expression of p65 and phospho-p65 in microglia, which provides a basis for the p65 acutely promoting expression of miR-124. We demonstrated that overexpression of p65 drove transcription of pri-miR-124-1 and pri-miR-124-3 other than pri-miR-124-2, in agreement with the prediction that transcriptional binding sites between p65 and miR-124 are located within pri-miR-124-1 and pri-miR-124-3 rather than pri-miR-124-2 promoters on genome loci. However, morphine exposure increased expression levels of all three pri-miR-124 in our study; thus, other transcriptional factors are likely to be involved in the transcription. Even though c-Fos was observed to be persistently increased after morphine treatment, overexpression of c-Fos did not induce miR-124 upregulation, similar to CREB, which are considered main transcription factors of morphine (31). Conclusively, our studies reveal that transcriptional factors p65, Ap-1, and CREB and cholinergic signaling are involved in regulation of miR-124 that exerts a central contribution to morphine inhibitory function on the innate immunity of microglia/macrophage through differential modulation ofTLRs and cholinergic signaling pathway (Supplemental Fig. 3).

In summary, our studies demonstrate that miR-124 plays a critical role in morphine-mediated suppression of microglia immunity, and upregulation of miR-124 is partially mediated by morphine-induced transient upregulation of p65. These findings provide direct molecular evidence for morphine-induced immune suppression.

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Disclosures

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