RESEARCH ARTICLE

Buprenorphine differentially affects M1- and M2-polarized macrophages from human umbilical cord blood

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ABSTRACT. ***Background:*** **As a partial μ-opioid receptor agonist with long half-life time, buprenorphine has been widely used to relieve chronic cancer and nonmalignant pain. The maintenance of chronic pain involves inflammation; however whether buprenorphine has anti-inflammation property remains unclear.** ***Methods:*** **Macrophages, the immune cells that initiate and maintain inflammation, were isolated from human umbilical cord blood, and were polarized into M1 or M2 macrophages with IFN-γ in the presence of lipopolysaccharide (LPS) or IL-4, respectively. Quantitative PCR, ELISA, Western blotting analysis, and chromatin immunoprecipitation assays were employed to characterize M1 and M2 macrophages.** ***Results:*** **1) Buprenorphine did not change not only the apoptosis, survival, and morphology of resting macrophages, but also the antigen-presenting function of macrophages. 2) Buprenorphine inhibited the levels of mRNA and protein of several cytokines in M1 macrophages, and enhanced the expression of Ym1 and Fizz1 in M2 macrophages. 3) Buprenorphine did not affect the modulation of NF-κB and MAPK cascades by LPS in M1 macrophages. 4) Buprenorphine inhibited the expression of IRF5 and reduced binding of DNA to IRF5.** ***Conclusion:*** **Buprenorphine may downregulate IRF5 pathway and limit M1 macrophage phenotype. These effects may contribute to its therapeutic benefit for chronic neuropathic pain.**

Key words: buprenorphine, macrophage, IRF5, downregulation, lipopolysaccharide

The opioid drugs are the primary options in the treatment of cancer pain [1, 2], and are among the drugs that effectively relieve chronic nonmalignant pain, including inflammatory, ischemic, visceral, musculoskeletal, and neuropathic pain [3, 4]. It is noteworthy that not every patient with chronic pain symptoms satisfyingly responds to opioids [5], and also long-term use of opioids causes the development of tolerance, dependence, and hyperalgesia [6, 7]. These facts limit their clinical use to treat chronic pain.

Buprenorphine is an opioid drug, acting as a weak partial agonist on μ-opioid receptors, but an antagonist on κ- and δ-opioid receptors [2, 8]. Having a slow onset and mild effect, buprenorphine is not suitable to treat acute pain and post-operative pain [9, 10]. However, it has been widely used in the management of chronic pain [11] because of its very long half-life time (24 to 60 hours) [10]. In comparison with other opioid drugs, buprenorphine has a series of advantages [12]: broader array of indications (cancer pain, neuropathic pain, etc.), a ceiling effect on life-threatening respiratory depression, minor cognitive impairment, less constipation, minimal effect on cardiovascular system, lower likelihood to develop drug dependence, etc.

Neuropathic pain not only involves neuronal dysfunction, but also activation of immune cells, which releases cytokines and chemokines, facilitating pain signaling [13]. Opioid receptors present in various types of immune cells [14], and morphine is demonstrated to inhibit natural killer (NK) cell activity, cytokine expression, chemokine induced chemotaxis, and phagocytic activity [15, 16]. However, buprenorphine has minimal direct effect on the immune system when there is no inflammatory stimulation [17, 18]. There is evidence supporting that buprenorphine alters the function of immune system in the presence of inflammatory stimulation. For instance, buprenorphine reduces immunosuppression following surgery in rodents [17]. In mice undergoing sepsis following cecal ligation and puncture, buprenorphine increased airway macrophage numbers, peripheral white blood cells, and total peripheral lymphocytes [19]. Considering anti-inflammation as a key strategy for neuropathic pain treatment, except for inhibiting neurons in pain transduction pathway through μ-opioid receptors, regulating inflammation could be another pathway contributing to its pain relief benefit.

Among immune cells, macrophages are the primary sensors of inflammatory stimulation, and might initiate neuropathic pain [13]. In the present study, we isolated macrophages from human umbilical cord blood and examined the direct effects of buprenorphine on them, including number, morphology, cytokine products, and underlying signal transduction pathways. We found that

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buprenorphine regulated macrophages when they were polarized into M1 state probably through a signaling pathway involving IRF5.

METHODS AND MATERIALS

Blood collection and storage

Eight subjects, enrolled in this study, were from the Department of Obstetrics and Gynecology in the Daqing Oil Field General Hospital. Every subject was aware of the fact that the umbilical cord blood was collected only for this study {CE: Please raise an AQ.}. All procedures were reviewed and approved by Institutional Review Board of Daqing Oil Field General Hospital.

Five ml blood was collected from umbilical cord, and was immediately stored at -80 °C. Before experiments, the samples were thawed at 37 °C, and were maintained at indicated temperatures.

Macrophage isolation using flow cytometry

Human umbilical cord blood was stored at 4 °C in the dark for 30 minutes, then, the red blood cells were lysed with lysing solution (Ortho Diagnostic), and were washed twice with phosphate buffer solution (PBS) containing 0.5% bovine serum albumin. The remaining white blood cells were cultured in high glucose DMEM at 37 °C in 5% CO_2 for 24 hours. As the macrophage progenitor cells in cultures adhere to the bottom of the culture dish, high purity of these cells was achieved by changing culture medium and washing away the floating cells. Then, the cells were detached, and were used for flow cytometry analysis. After detachment, the cells were incubated with PE-conjugated anti-CD68 (eBioscience, BM8, San Diego, CA, USA) and APC-conjugated anti-CD11b (eBioscience, M1/70, USA). Flow Cytometry (FACSAriaII, BD Biosciences, Franklin Lakes, NJ, USA) was employed to sort cells positive for both CD11b and CD68, representing macrophages.

Macrophage cell suspension was plated in culture dish containing high glucose DMEM to obtain a cell density of 2×10^5 cells per dish, and was incubated at 37 °C in 5% CO_2 [20]. Five days later, the cultured cells were subject to starvation overnight, and then, those were exposed to LPS (100 ng/mL) and IFN-γ (10 ng/mL), or IL-4 (10 ng/mL). The exposure duration was 0, 2, and 6 hours for qPCR assay, and 2 and 24 hours for ELISA. Buprenorphine was added into the medium at 0.8 or 4 ng/mL together with LPS + IFN-γ or IL-4. The morphological information was acquired with a phase-contrast inverted microscope (Olympus, IX71, Tokyo, Japan).

Apoptosis assay with Annexin-V-FITC/PI double staining

The cultured macrophages (2×10^5) were detached with 0.25% trypsin, which was then terminated with 2% BSA. The macrophages were collected by centrifugation (2000 rpm for 5-10 min) at room temperature, and then those were washed with cold PBS (4 °C) and centrifuged again for 5-10 min, and 300 μL binding buffer was added to suspend the cells. 5 μL Annexin V-FITC was added and mixed, then incubated at room temperature in the dark for 15 min. 5 μL PI staining reagent was added 5 min before Flow Cytometry analysis, and the mixture was diluted by adding 200 μL binding buffer just before assay.

Quantitative polymerase chain reaction (qPCR)

Macrophages were incubated in high glucose DMEM, and collected 2 and 6 hours after adding buprenorphine (0.8 and 4 ng/mL) and vehicle. Total RNA in macrophages was acquired, and then complementary DNA libraries were generated using a commercially available kit (Qiagen, Valencia, CA, USA). The qPCR master mixture was purchased from Qiagen. After adding cDNA samples and pairs of primers in the master mixture, qPCRs were run for 40 cycles in a thermocycler (BioRad, Hercules, CA, USA). The primers of q-PCR were shown in *table 1*. The relative expression level for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method [21] and all PCR values were normalized to those of β-actin.

Enzyme-linked immunoabsorbent assay (ELISA)

Macrophages were incubated in DMEM. Before experiments, the medium was freshly changed, and was collected immediately or 24 hours after adding vehicle and

Table 1
q-PCR-primers

Gene	forward	Reverse
IL-6	CTTCGGTCCAGTTGCCTTCT	TGGAATCTTCTCCTGGGGGT
TNF-α	TGGGGAGTGTGAGGGGTATC	TGCACCTTCTGTCTCGGTTT
IL12a	TTCGCTTTCATTTTGGGCCG	ATCAGCTTCTCGGTGACACG
IL12b	AGAACTTGCAGCTGAAGCCA	CCTGGACCTGAACGCAGAAT
Arg1	GGAAGTGAACCCATCCCTGG	CGAGCAAGTCCGAAACAAGC
Ym1	AGGAGGCCAAGCAGATCAAC	TAGAGGGGGCTGTTCTCTCC
Mrc1	GCCAACAACAGAACGCTGAG	ACACTTGTGCTGTTGACTTCT
Fizz-1	GTCAAAAGCCAAGGCAGACC	TGAACATCCCACGAACCACA
IL-10	CAGCTCAGCACTGCTCTGTTG	CTCCAGCAAGGACTCCTTTAAC
KLF4	CGAACCCACACAGGTGAGAA	TACGGTAGTGCCTGGTCAGTTC
β-actin	CTACAATGAGCTGCGTGTGG	AAGGAAGGCTGGAAGAGTGC

buprenorphine (0.8 and 4 ng/mL). Cytokines, including TNF-α, IL-12, and IL-6, in each sample were measured with sandwich ELISA [22]. Briefly, antibodies of these cytokines were non-covalently adsorbed onto 96-well plastic plates for 2 hours. After washing out free antibodies, the culture medium was applied to the plate, and incubated over-night at 4 °C. The solution was exchanged with PBST for 3 times (5 min each). Then, biotin-conjugated anti-cytokine antibodies were added to bind to each cytokine, followed by adding horseradish peroxidase-labeled avidin or streptavidin. ABC HRP kit (MultiSciences) was used for generating color, which was measured with a spectrophotometer (PerkinElmer).

Chromatin immunoprecipitation (ChIP) assays

After 5 days in culture, the macrophages (2 × 106) were subject to starvation overnight (12 hours), and then those were stimulated with LPS for 6 hours. After these procedures, the macrophages were further processed to obtain cellular contents: fixation with 1% formaldehyde in culture medium at 37°C for 10 min, rinse with PBS, detachment from the culture dish, centrifugation to harvest cells, and addition of lysis buffer with proteinase inhibitor to break cell membrane, followed by sonication for the fragmentation of large molecules, including DNA. The lysates were centrifuged (at 10,000 g, 10 min) at 4°C, and the supernatant was collected, was heated at 65°C for 3 hours, was mixed with ChIP dilution buffer and protein A agarose/Salmon Sperm DNA in a rotation apparatus at 4°C for 1 hour, and was kept stationary for 10 min to eliminate precipitation. The supernatant was incubated with IRF5 antibody on a shaker overnight at 4°C. The reaction product was incubated with protein A agarose/Salmon Sperm DNA at 4°C for 2 hours, and then centrifuged to collect precipitates, which were subsequently rinsed and eluded to acquire samples containing IRF5 and its binding DNA. After incubation at 65°C overnight, the DNA in the samples was dissociated with proteins, and then, the DNA was purified by Qiaquick columns (Qiagen, USA), and was quantified by qPCR using a pair of primers that amplify IL-6 promoter. The precipitated DNA is presented as percentage of the total input DNA.

Statistical analysis

All the statistical analyses were performed by one- or two-way ANOVA analysis followed by a Tukey's post hoc test using SPSS software. All the data points were presented as mean ± S.E.M from at least 3 individual experiments. P value less than 0.05 was considered as significant difference.

RESULTS

Buprenorphine did not alter the apoptosis of macrophages in vitro

To understand whether buprenorphine has toxic or protective effects on macrophages, we performed Annexin-V-FITC and PI double staining to count cells undergoing early and late apoptosis, respectively. We did not observe any significant effects of buprenorphine (0.8 and 4 ng/mL) on apoptosis of macrophages (*figure 1A*). Buprenorphine slightly increased the viability of macrophages in 12 hours, but without statistical significance (*figure 1B*). Additionally, up to 24 hours in *in vitro* cultures, macrophages showed no morphological change in the presence of buprenorphine (*figure 1C*). The data suggest that buprenorphine may not cause or prevent apoptosis of macrophages.

Buprenorphine does not change cell surface expression of co-stimulatory molecules on macrophage

To test whether buprenorphine changes the function of macrophages, we exposed macrophages to LPS to induce antigen-presenting process, and evaluated the effects by measuring the expression of surface co-stimulation molecules, such as, CD80, CD86, and MHC II (*figure 2A*). Our data showed that buprenorphine did not affect these surface co-stimulation molecules (*figure 2B*), suggesting that buprenorphine did not impair antigen-presenting function in macrophages.

Buprenorphine differentially affects M1 and M2 macrophages

We further examined the expression and release of cytokines after macrophages were incubated with IFN-γ + LPS or IL-4, which are assumed to polarize macrophages into M1 and M2 states, respectively. As illustrated in *figure 3A*, IFN-γ + LPS time-dependently elevated the mRNA of TNF-α, IL-12a, IL-12b, and IL-6, the cytokines released by M1 macrophages; buprenorphine significantly inhibited the expression of these cytokines. We then performed ELISA to measure the released cytokines in the incubation medium. Data in *figure 3B* showed similar changes in the protein levels of TNF-α, IL-12, and IL-6 in the presence of IFN-γ + LPS with and without buprenorphine. We also examined iNOS (*figure S1A*), another molecule in M1 macrophages, but exposure to buprenorphine for 2 and 6 hours did not change its level. Furthermore, we prepared macrophages from mouse peripheral blood, and found that buprenorphine similarly inhibited TNF-α and IL-6 in M1 macrophages (*figure S2*).
When macrophages were polarized into M2 state by IL-4, we observed time-dependent elevation of mRNAs of Arg1, Ym1, Mrc1, and Fizz1, and buprenorphine further enhanced mRNA levels of Ym1 and Fizz1 (*figure 3C*). Although IL-10 and KLF4 showed time-dependent expression in M2 macrophages, but buprenorphine exerted no effect on these molecules (*figure S1B*).
These data suggest that buprenorphine affects inflammatory responses of macrophages: inhibiting cytokine expression and release from M1 macrophages, but enhancing the expression of healing molecules of M2 macrophages. Interestingly, buprenorphine did not uniformly alter featured molecules in either M1 or M2 macrophages.

Buprenorphine inhibits IRF-5 expression but not NF-κB and MAPK cascades

We next examined signaling cascades that are recruited when macrophages respond to the stimulation by LPS, a toll-like receptor-4 (TLR-4) agonist. We performed Western blotting analysis to test the effects of LPS on the molecules involved in NF-κB and MAPK cascades in macrophages (*figure 4A-C*). We observed that LPS

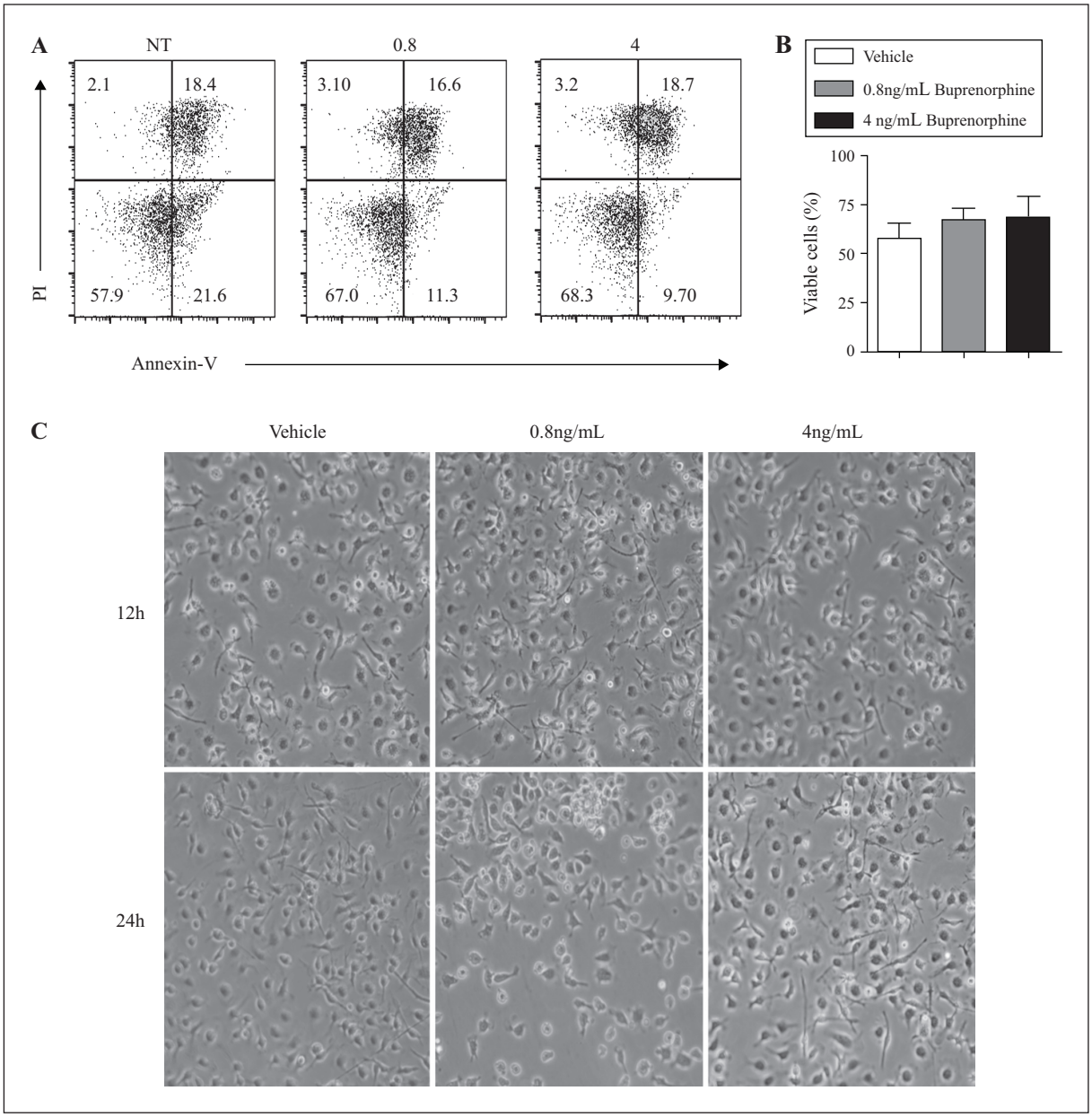

Figure 1

Buprenorphine does not change the apoptosis and morphology of macrophages. **A)** Cells were stained with PI and Annexin V-APC. Thin lines represent vehicle and thick lines represent stimulations as indicated. **B)** Bar graphs show percentage of viable cells in different conditions. **C)** Morphology of macrophages isolated from human umbilical cord blood cultured for 12 h and 24 h with different doses of buprenorphine. Under an inverted microscope in the phase-contrast mode, Magnification: 400×.

enhanced all signal cascades mentioned above. In the NF-κB signaling cascade, it time-dependently promoted phosphorylation of IKKα/β, but downregulated the phosphorylation of IKBα, and increased the expression of C/EBP-β, C-Rel, P50, and P65 (*figure 4A, B*). In MAPK cascades, LPS enhanced phosphorylation of JNK, P38, and ERK1/2 (*figure 4C*). Co-application of buprenorphine with LPS caused similar changes in these cascades to LPS alone, suggesting that buprenorphine affects M1 macrophages probably through signaling pathways other than NF-κB and MAPK cascades.

We then tested LPS effects on IRF1 and IRF5, two transcription factors in M1 macrophages, and observed that LPS increased the expression of IRF1 but did not change the expression of IRF5 (*figure 4D*). Addition of buprenorphine significantly decreased IRF5 expression, but left LPS modulation of IRF1 intact (*figure 4D*). The reduction of IRF5 was also supported by our data that buprenorphine decreased IRF5 mRNA levels (*figure 4F*). We performed CHIP to test whether buprenorphine impaired the ability of IRF5 to bind DNA. We used IRF5 antibody to precipitate IRF5 and its binding molecules from macrophage lysis. As IRF5 is a transcription factor that regulates the expression of IL-6, measuring the amount of IL-6 DNA could assess the binding capability of IRF5. After IRF5 binding DNA was collected, we quantified IL-6 promoter with qPCR. We found that buprenorphine dramatically reduced the binding of IRF5 with IL-6 promoter (*figure 4E*). These results suggest that buprenorphine downregulated signaling pathways involving IRF5.

DISCUSSION

Macrophages are widely present in all body tissues, and are easily polarized into two functionally distinct

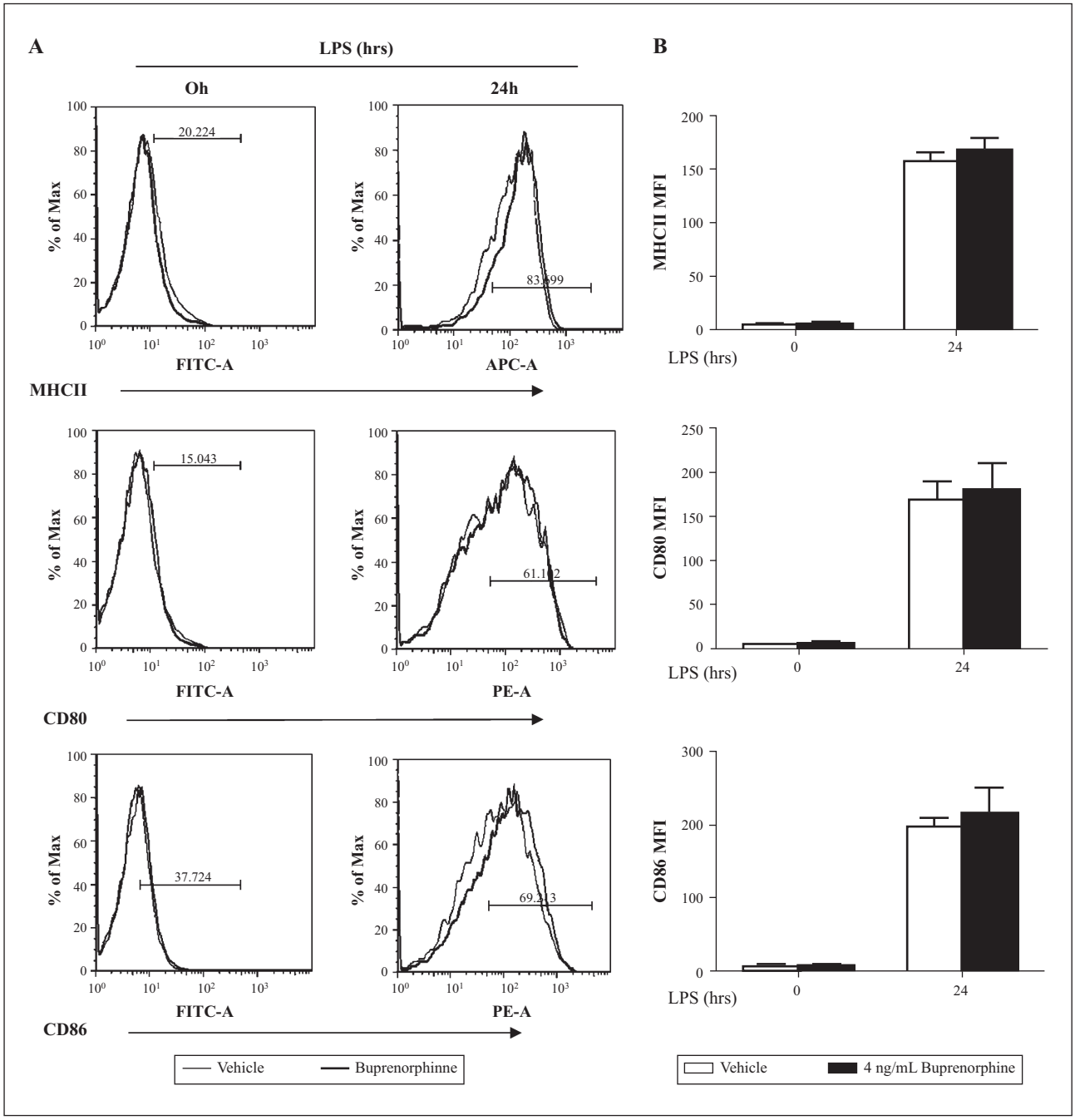

Figure 2

Levels of surface co-stimulation on LPS-stimulated macrophages. Representative plot **(A)** and a summary graph of mean fluorescence intensity (MFI, **B**). Data are presented as mean ± SEM of values from at least three independent experiments.

subpopulations, named M1 and M2, in response to various environmental stimuli [23, 24]. IFN-γ or LPS preferentially polarizes resting macrophages into an M1 type, enhances their antigen-presenting phenotype, and initiates or exacerbates inflammation [23-26]. When macrophages are polarized into an M2 type by IL-4 [23, 27], they act as suppressors of inflammation, and facilitate the healing process [23, 24]. The profile of cytokines, chemokines, and some other molecules in macrophages changes when these cells are skewed into an M1 or M2 type. M1 macrophages express some pro-inflammation factors, namely IL-6, TNF-α, IL-12, etc. [23, 28], while M2 macrophages produce some particular molecules, including resistin-like-α (Fizz1), arginase1 (Arg1), chitinase 3-like 3 (Ym1), and Mrc1, etc. [23, 29]. The macrophages can alternate between these two types and can also be converted into resting state which involves IRF/STAT signaling pathways [24]. Higher IRF5 levels are one of characteristics of M1 macrophages, and IRF5 is downregulated when macrophages are polarized into M2 state [30]. In the present study, we explored the effects of buprenorphine on M1 and M2 macrophages.

We isolated macrophages from human umbilical cord blood, because these cells have at least two advantages. First, these cells provide *in vitro* system to test the direct effects of buprenorphine on macrophages without being interfered by its modulation of opioidergic, serotonergic and noradrenergic systems in the central nervous system.

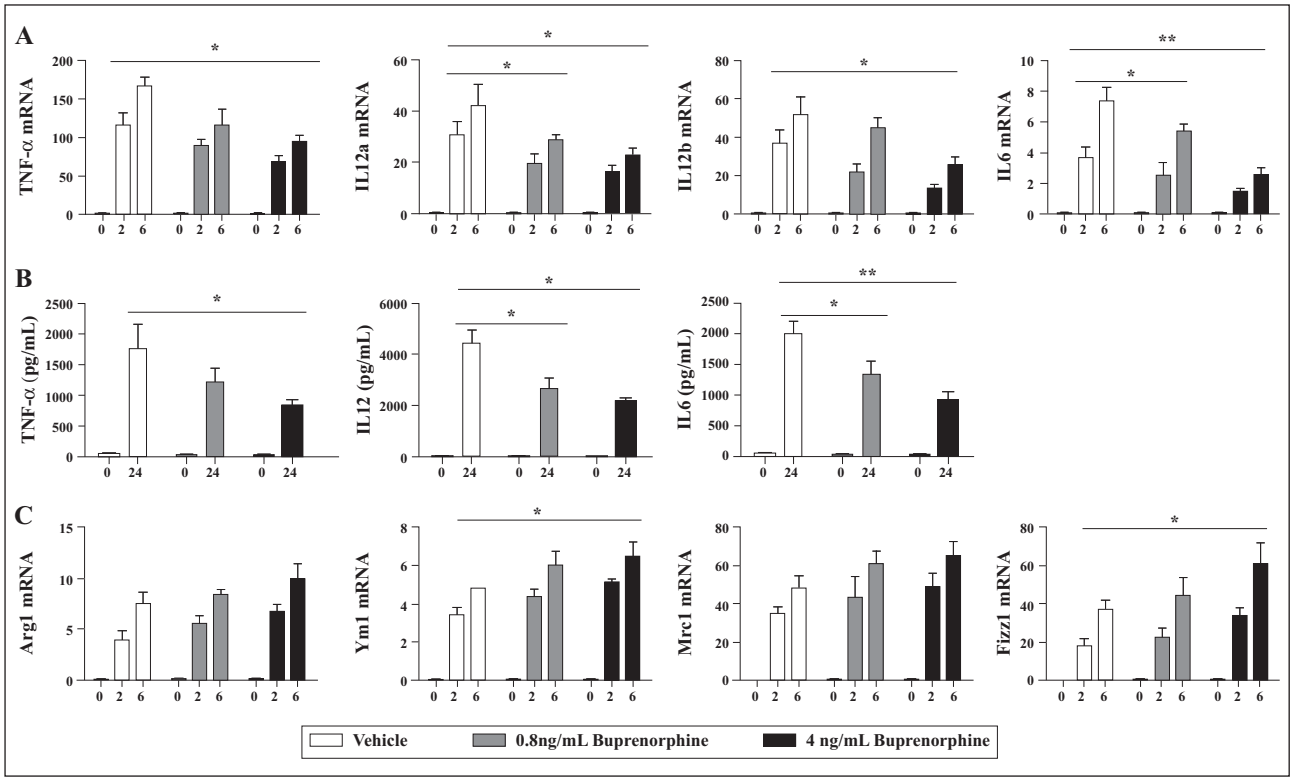

Figure 3

Buprenorphine regulated levels of distinct cytokines and molecules in M1 and M2 macrophages. qRT-PCR **(A)** and ELISA **(B)** assessment of several cytokines in macrophages 0, 2 and 6 hours after IFN-γ and LPS stimulation without (vehicle) and with buprenorphine (0.8, 4 ng/mL). **(C)** qRT-PCR analysis of the indicated genes in macrophage 0, 2 and 6 h after IL-4 stimulation without (vehicle) and with buprenorphine (0.8, 4 ng/mL). For qPCR, the mRNA levels are presented as folds of those of Actin; For ELISA, data are presented as folds relative to Actin levels. Data are presented as mean ± SEM from at least three independent experiments. *P<0.05; **P<0.01.

Second, these cells are protected by blood placenta barrier from exposure to various cytokines and drugs in maternal circulation. Therefore, the macrophages are mostly at the resting state and, after being isolated, they can be preferentially polarized into M1 and M2 types with an incomparable purity that the macrophages from maternal circulating blood can have.

After confirming that in our culturing condition, macrophages did not show accelerated apoptosis and death, and maintained consistent morphology, we tested the effects of buprenorphine on apoptosis (*figure 1*) and antigen presenting function (*figure 2*) of macrophages *in vitro*. Our data indicated that buprenorphine did not significantly alter these parameters. However, buprenorphine differentially regulated the synthesis of cytokines or reaction molecules which exist in M1 and M2 macrophages. In M1 macrophages, it reduced the expression (mRNA) and release (protein) of several cytokines, including IL-6, TNF-α, IL-12 (*figure 3*), but not iNOS (*figure S1*). Buprenorphine had similar effects on mouse M1 macrophages (*figure S2*), indicating that its effects were not specific to human macrophages. In contrast, buprenorphine elevated the expression of Ym1 and Fizz1. The results suggest that buprenorphine may inhibit inflammation initiation and maintenance by reducing cytokine levels released from M1 macrophages, but facilitate healing processes by promoting the expression of Ym1 and Fizz1 in M2 macrophages.

As inflammatory cytokines facilitate pain signaling and trigger hypersensitivity to nociceptive stimulation, they play important roles in the development and maintenance of neuropathic pain [13]. Although buprenorphine is recognized as an effective treatment for neuropathic pain [10, 11], its weak opioidergic action [9, 10] prompts a notion that it may exert its therapeutic effects through other pathways. Our data support this notion, and reveal that controlling the inflammatory reaction could be a vital aspect underlying the benefits of buprenorphine to neuropathic pain.

LPS modulates macrophages through NF-κB and MAPK cascades [31]. Before activation, NF-κB binds to its inhibitor, IκB. Following phosphorylation of IKKbα/β, then subsequent phosphorylation of IκB, IκB dissociates from NF-κB, resulting in the activation of NF-κB pathway [32]. In the present study, we examined several NF-κB proteins, including p50, p65, and c-Rel. Consistent with previous studies [23, 24], we observed that LPS upregulated phosphorylation of IKKα/β, but downregulated the phosphorylation of IKBα, increased expression of C/EBP-β, C-Rel, P50 and P65. We also examined the mobilization of MAPK cascades by LPS. It increased phosphorylation of JNK, P38, and ERK1/2, suggesting the activation of these cascades (*figure 4C*). But buprenorphine caused no changes in LPS-effects on these two cascades.

Therefore, the results in *figure 4A-C* suggest that buprenorphine affects the function of macrophages via pathways other than NF-κB and MAPK cascades. But this needs to be clarified with further investigations. As a matter of fact, we tested buprenorphine effects on two cascades after

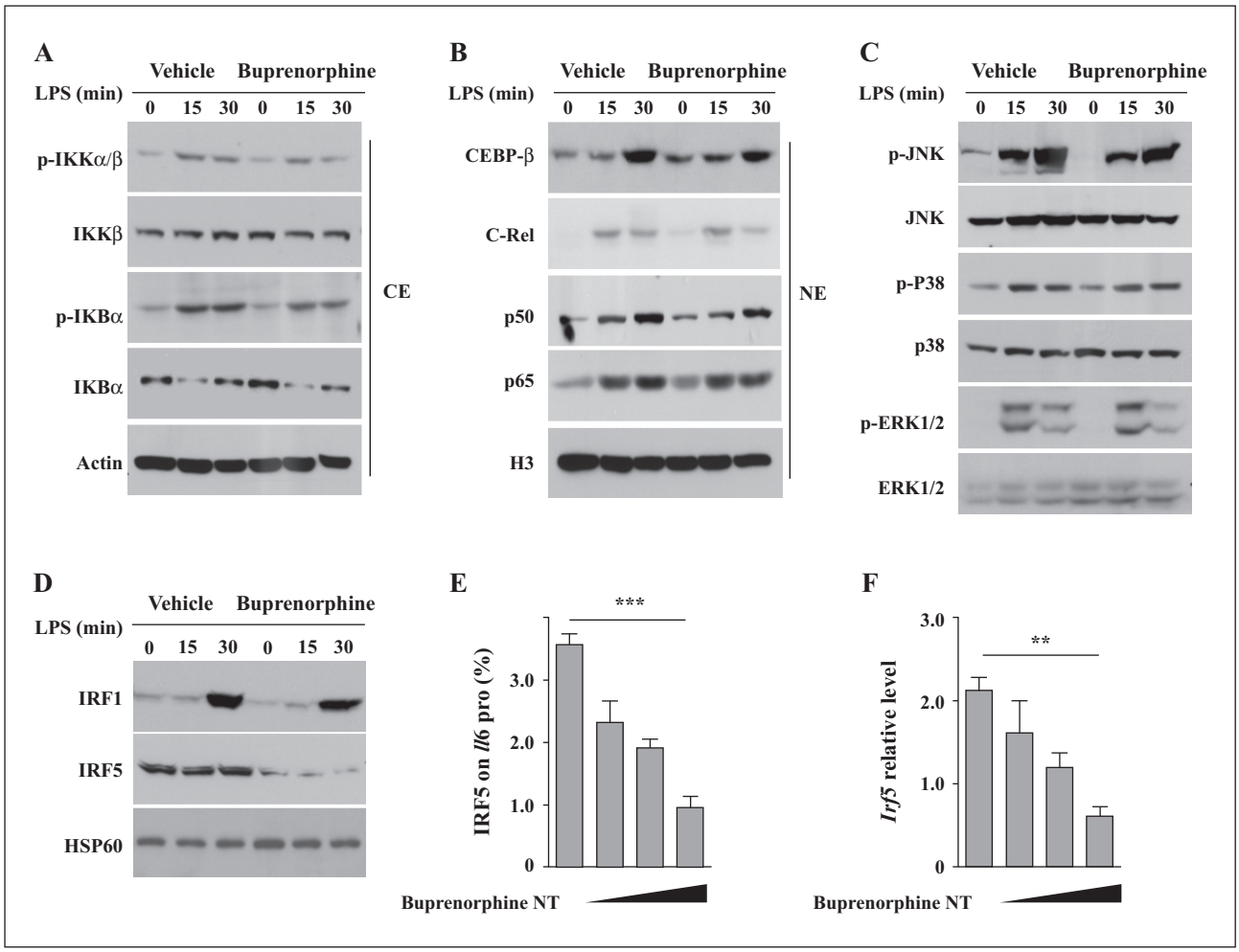

Figure 4

Buprenorphine downregulated the IRF5 pathway but did not alter NF-κB and MAPK cascades in macrophages**. A-D)** Several molecules in NF-κB and MAPK cascades were analyzed with western blot assay. The phosphorylated forms of these molecules were semi-quantified relative to their total protein levels. The effect of LPS and LPS + buprenorphine (4 ng/mL) on these molecules were examined. **E)** ChIP assays to detect the binding of IRF5 to the IL-6 promoters upon 6 hours LPS stimulation without and with buprenorphine (4 ng/mL). Data are presented as percentage based on total input DNA quantified by qPCR. Data are presented as mean ± SEM of the percentage of IRF5-bound DNA over total input DNA. Statistical analyses represent variations in technical replicates. **F)** qRT-PCR analysis of the IRF5 mRNA level in macrophages treated with different doses of buprenorphine. **P<0.01, ***P<0.005.

15 and 30 min (*figure 4A-C*), while we tested its effects on cytokine expression and release in M1 macrophages and M2 macrophage specific proteins 2-24 hours after the exposure to buprenorphine. To confirm the involvement of these two cascades in buprenorphine effects on M1 macrophages, two sets of experiments should be performed at the same time points after exposure to buprenorphine.

As IRFs play pivotal roles in the alternation of macrophages between M1 and M2 states, we hypothesized that it might be one of the targets of buprenorphine to limit pro-inflammatory effects of M1 macrophages. We observed that LPS induced an enhancement of IRF1, but did not change IRF5. Although buprenorphine did not change LPS-effects on IRF1, it dramatically reduced IRF5 (*figure 4D*). The reduction of IRF5 protein is consistent with the attenuation of IRF5 mRNA shown in *Figure 4F*. The CHIP assay revealed that in addition to reduce expression levels of IRF5, buprenorphine also impaired its DNA binding ability. The weakened function of IRF5 in macrophages in the presence of buprenorphine suggests that buprenorphine may tend to limit the polarization of macrophages to M1 state.

CONCLUSION

In summary, buprenorphine did not alter the phenotype resting macrophages and their antigen-presenting function in response to LPS, but differentially regulated M1 and M2 macrophages. Buprenorphine attenuated LPS-induced cytokine production and secretion in M1 macrophages, but facilitated the production of inflammation-responding molecules synthesized in M2 macrophages. Although NF-κB and MAPK cascades importantly mediate LPS effects on macrophages, buprenorphine did not alter these pathways. Interestingly, buprenorphine downregulated the IRF5 pathway, which may limit the phenotype of M1 macrophages. The effects of buprenorphine on macrophages could be a potential mechanism underlying its benefits in neuropathic pain.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1684/ecn.2017.0392

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