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Buprenorphine Decreases the CCL2-Mediated Chemotactic Response of Monocytes

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Despite successful combined antiretroviral therapy, ~60% of HIV-infected people exhibit HIV-associated neurocognitive disorders (HAND). CCL2 is elevated in the CNS of infected people with HAND and mediates monocyte influx into the CNS, which is critical in neuroAIDS. Many HIV-infected opiate abusers have increased neuroinflammation that may augment HAND. Buprenorphine is used to treat opiate addiction. However, there are few studies that examine its impact on HIV neuropathogenesis. We show that buprenorphine reduces the chemotactic phenotype of monocytes. Buprenorphine decreases the formation of membrane projections in response to CCL2. It also decreases CCL2-induced chemotaxis and mediates a delay in reinsertion of the CCL2 receptor, CCR2, into the cell membrane after CCL2-mediated receptor internalization, suggesting a mechanism of action of buprenorphine. Signaling pathways in CCL2-induced migration include increased phosphorylation of p38 MAPK and of the functional protein JAM-A. We show that buprenorphine decreases these phosphorylations in CCL2-treated monocytes. Using DAMGO, CTAP, and Nor-BNI, we demonstrate that the effect of buprenorphine on CCL2 signaling is opioid receptor mediated. To identify additional potential mechanisms by which buprenorphine inhibits CCL2-induced monocyte migration, we performed proteomic analyses to characterize additional proteins in monocytes whose phosphorylation after CCL2 treatment was inhibited by buprenorphine. Leukosialin and S100A9 were identified and had not been shown previously to be involved in monocyte migration. We propose that buprenorphine limits CCL2-mediated monocyte transmigration into the CNS, thereby reducing neuroinflammation characteristic of HAND. Our findings underscore the use of buprenorphine as a therapeutic for neuroinflammation as well as for addiction. The Journal of Immunology, 2015, 194: 3246–3258.

An estimated 34 million people live with HIV worldwide (1). HIV enters the brain early after peripheral infection (2). Despite the success of combined antiretroviral therapy in reducing viral load, 40–70% of the HIV-infected population develop cognitive, behavioral, and motor deficits characteristic of HIV-associated neurocognitive disorders (HAND). The prevalence of HAND continues to rise as individuals with HIV live longer (3–6). Infected monocytes transport virus into the CNS, resulting in infection of macrophages and microglia, and to a lesser extent astrocytes. These cells produce cytokines, chemokines, and viral proteins, leading to recruitment of additional monocytes into the brain, neuroinflammation, and eventual neuronal damage (7–10). Thus, the transmigration of HIV-infected and uninfected monocytes into the CNS plays a key role in the initiation and progression of HAND.

Monocyte transmigration across the blood brain barrier (BBB), mediated in part by the chemokine CCL2, is critical to the neuropathogenesis of HIV (11–13). CCL2 is the most potent monocyte chemoattractant (14, 15) and is highly elevated in the brain tissue and CSF of people with HAND (16). Even with successful antiretroviral therapy, CCL2 levels in the CNS of infected people remain elevated, resulting in ongoing monocyte transmigration into the brain and chronic, low-level neuroinflammation (17–19). Cognitive impairment correlates closely with the extent of neuroinflammation and with CCL2 in the CNS (16, 20). CCL2 is released by infected monocytes, macrophages, microglia, and astrocytes, promoting monocyte entry into the CNS (21, 22). Our laboratory previously demonstrated that HIV-infected monocyte transmigration across the BBB in response to CCL2 is significantly higher when compared with uninfected monocytes and that CCL2 transiently disrupts BBB integrity (22–24).

Many studies indicate that HIV-infected opiate abusers exhibit increased CNS inflammation, neuronal injury and death, and neuropathology as compared with HIV-infected nondrug users, all of which contribute to cognitive impairment (25–27). Following acute withdrawal from opiates, people can be maintained on a full opioid agonist, methadone, or a partial opioid agonist, buprenor-
phine. Buprenorphine is a partial agonist of the μ-opioid receptor and an antagonist of the κ-opioid receptor. It can be given at higher doses than methadone with fewer adverse effects. Buprenorphine increases the duration of both opioid withdrawal suppression and opioid blockade (28–31). However, the effects of buprenorphine on the mechanisms that mediate neuroinflammation and cognitive impairment during HIV infection have not been examined. Previous studies showed that agonists of specific opioid receptors, including the μ-opioid receptor agonist [d-Ala2-N-Me-Phe4, Gly-o15]-enkephalin (DAMGO) and the δ-opioid receptor agonist [d-Pen2, d-Pen5]-enkephalin, suppress the migration of both neutrophils and monocytes in response to multiple factors, including chemokines (32–34). Thus, we propose that buprenorphine decreases CCL2-induced monocyte migration by decreasing surface and cytoskeletal protein rearrangements, as well as intracellular signaling pathways that are necessary for these cells to transmigrate into the CNS in response to CCL2. This would result in decreased neuroinflammation and less neuronal damage in HIV-infected opioid abusers being treated with buprenorphine, and would therefore be an additional clinical benefit of this therapeutic drug.

Materials and Methods

Materials

Buprenorphine, DAMGO, H-D-Phe-Cys-Tyr-D-Asp-Arg-Thr-Pen-Thr-NH2 (CTAP), nor-binaltorphimine (Nor-BNI), acetone, DTT, and iodoacetamide were from Sigma-Aldrich (St. Louis, MO), and CCL2 was from R&D Systems (Minneapolis, MN). NaODSO4 (SDS) was from Bio-Rad (Hercules, CA), urea from GE Healthcare (Piscataway, NJ), and sequencing grade modified trypsin from Promega (Madison, WI), and detergent removal spin columns, trifluoroacetic acid, and formic acid were from Pierce (Rockford, IL). Tiitanum Phos-TIO Kit was from GL Sciences (Torrance, CA), isobaric tags for relative and absolute quantitation (iTRAQ) reagent 4plex kit was from AB Sciex (Foster City, CA), urea from GE Healthcare (Piscataway, NJ), and detergent removal spin columns, trifluoroacetic acid, and formic acid were from Pierce (Rockford, IL). Tiitanum Phos-TIO Kit was from GL Sciences (Torrance, CA), isobaric tags for relative and absolute quantitation (iTRAQ) reagent 4plex kit was from AB Sciex (Foster City, CA), and liquid chromatography/mass spectrometry grade acetonitrile and water were from Fisher Scientific (Waltham, MA).

Cell isolation

Leukopaks were obtained from the New York Blood Center in accordance with Albert Einstein College of Medicine guidelines. PBMC were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden), density gradient centrifugation. Monocytes were isolated from PBMC by positive selection using the CD14 EasySep selection kit (Stem Cell Technologies, Vancouver, BC, Canada), according to the manufacturer’s protocol.

Immunofluorescence

Human monocytes were treated with 200 ng/ml CCL2 or an equal volume of 0.1% BSA in PBS, the CCL2 diluent, for 15 min. Cells were then fixed with 2% paraformaldehyde in PBS for 1 h, permeabilized with 0.01% Triton, and incubated in blocking solution containing 0.5 M EDTA, 1% fish gelatin, 1% Ig-free BSA, 1% horse serum, and 1% human serum for 30 min. After blocking, cells were stained with anti-tubulin Ab (1:10,000, T9026; Sigma-Aldrich) for 1 h at room temperature. The cells were washed and then incubated with the secondary Ab, anti-mouse IgG F(ab’)2 fragment-FITC (1:100, F2883; Sigma-Aldrich) mixed with Texas Red-X phalloidin (15 μM/ml, T7471; Invitrogen, Grand Island, NY) for 2 h at room temperature. Samples were washed and then mounted in Prolong Gold anti-fade reagent (P36931; Invitrogen) and examined by confocal microscopy using a Leica SP2 confocal microscope.

Chemotaxis assay

Cell migration assay was performed using a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD). A total of 3 × 105 monocytes was incubated for 15 min at 37°C in the presence of BSA (CCL2 diluent), buprenorphine (20 nM), CCL2 (200 ng/ml), or buprenorphine plus CCL2. Cells were then washed, resuspended in 50 μl RPMI 1640 plus 2% FBS, and placed in the upper well of the microchemotaxis chamber, separated from the lower well by a polycarbonate filter with 5-μm pores (Neuro Probe). The chemotactic response of monocytes was assayed after the addition of CCL2 or BSA diluted in RPMI 1640 to the lower well. The chamber was incubated at 37°C with 5% CO2 for 25 min. For CCR2 recovery experiments, monocytes were pretreated using the same protocol above, and, after 15 min of treatment, the cells were washed and incubated at 37°C with 5% CO2 for an additional 30 min. Cells were then added to the top well of the microchemotaxis chamber and allowed to migrate, as described above. Filters were then removed, fixed, and stained using Diff-Quick Stop Set (Siemens Healthcare Diagnostics,业务-A, JAMA-A-1:500, sc-17430-R; Santa Cruz Biotechnology, Dallas, TX), and JAM-A (1:500, sc-53623; Santa Cruz Biotechnology) overnight at 4°C with gentle rocking. After washing, membranes were incubated with either anti-rabbit HRP (1:2500, 7074; Cell Signaling), or anti-mouse HRP (1:2500, 7076; Cell Signaling), and immunocomplexes were detected by Western Lightning Chemiluminescence Reagent (PerkinElmer, Waltham, MA). Densitometric analyses were performed with UN-SCAN-IT gel digitizing software (Silk Scientific).

Proteomic analysis

Preparation of iTRAQ-labeled membrane protein phosphopeptides. Each experiment was performed with cells isolated from one leukopak. Membrane proteins were enriched from 20–30 × 10⁶ monocytes for each experimental condition using differential detergent fractionation with a Proteome Cell Compartment Kit from Qiagen (Valencia, CA) (41). After acetone precipitation, the membrane proteins were solubilized in 1% SDS, 8 M urea, 20% acetonitrile, and 50 mM TEAB (pH 8.5), followed by reduction with DTT and alkylation with iodoacetamide. A solution of 50 mM TEAB buffer (pH 8.5) containing 2 mM CaCl2 and 10% acetonitrile was added to reduce the concentration of SDS to 0.1% before adding trypsin (2.5 ng/μl) at times 0 and 4 h of an 12- to 14-h incubation at 37°C. SDS was removed by using 2 ml detergent removal spin columns (Pierce, Rockford, IL) before acidifying the sample with 20% trifluoroacetic acid to a final pH between 2 and 3 and concentrating on C18 spin columns con-
taining 42 mg C18 resin. After elution, tryptic peptides were prepared for iTRAQ labeling according to the manufacturer’s instructions (AB Sciex). Aliquots of the labeled samples were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) to verify completion of iTRAQ labeling. Samples from all the experimental conditions were pooled after confirming that no unlabeled peptides were identified. Phosphopeptides were enriched from the pooled iTRAQ-labeled sample with a TiO₂ column (GL Sciences, Rolling Hills Estates, CA), according to the manufacturer’s instructions, and as described elsewhere (42).

LC-MS/MS and data analysis. The enriched iTRAQ-labeled phosphopeptides were separated online by 2D-LC-MS/MS using a Waters NanoAcquity nano-UPLC system (Milford, MA) interfaced to an Orbitrap Velos high-resolution mass spectrometer (Thermo Scientific, San Jose, CA), as previously described (42). Text files were created from the raw data using Proteome Discoverer 1.2 (Thermo Scientific), merged, and searched against a Uniprot database of human and mouse protein sequences using the Mascot Protein Search Engine (Matrix Science, Boston, MA). The following parameters for searches were as follows: trypsin 2 missed cleavages; fixed modifications of carbamidomethylation (Cys), oxidation (Met); monoisotopic variable modifications of phosphorylation (Ser, Thr, and Tyr), iTRAQ 4plex (Tyr), and oxidation (Met); monoisotopic masses; peptide mass tolerance of 20 ppm; and product ion mass tolerance of 0.1 Da. A peptide mass error within 7 ppm and product ions within ± 0.05 Da were achieved; in the rare case of LC-MS/MS runs, the accurate observed peptide mass can be determined by an adjustment with the systematic mass error obtained from the same dataset. A false discovery rate (FDR) for peptide identification was assessed by decoy database searching. Proteins were considered identified having at least two bold red peptides unique to that sequence (significance p < 0.05). This method identifies the most logical assignment of a peptide to a specific protein, and prevents duplicate homologous proteins from being reported. The peptide FDR was <1%. Scaffold Q+ was used to validate MS/MS-based peptide and protein identifications and to quantify isobaric tag peptides and proteins (version 3.6.2; Proteome Software, Portland, OR). FDR was controlled under 0.1%. Intensity-based normalization was used for quantitation based on the median ratio and using the individual spectrum’s iTRAQ reporter ion as reference. Only uniquely assigned peptides were quantified. Differential expression was presented as log₂ fold change of reference. A change of 0.6 was considered significant. This corresponds to a ~1.5-fold difference in expression. Phosphoproteins that were common to all three donors were selected for Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA). Unphosphorylated peptides from the same peptide mixture were also analyzed to evaluate the stoichiometry of phosphorylation.

Results

Buprenorphine reduces the CCL2-induced migratory phenotype in monocytes

CCL2 is elevated in the CNS of people with HAND and mediates increased monocyte recruitment and transmigration into the brain (16, 20). Monocyte movement is necessary for entry into the CNS and is associated with cytoskeletal changes, including actin and tubulin reorganization, that regulate locomotion and chemotactic migration (43–45). Monocytes express opioid receptors and therefore are able to respond to buprenorphine (46–49). Sublingual doses of 8 mg buprenorphine once or twice daily are used to treat drug addiction, giving peak plasma concentrations of 10–20 nM (50–52). Therefore, we chose 20 nM buprenorphine to examine whether this therapeutic concentration impacts the migratory phenotype induced in monocytes by CCL2.

Human monocytes isolated from PBMC were treated with 200 ng/ml CCL2, an equal volume of 0.1% BSA in PBS (CCL2 diluent), buprenorphine, or CCL2 plus buprenorphine for 15 min, and stained for actin and tubulin. Immunofluorescence was examined by confocal microscopy, and the percentage of cells with membrane projections was quantified as an indicator of a CCL2-induced migratory phenotype (43, 44). In control conditions (BSA), the monocytes were spherical and actin was distributed throughout the cell body (Fig. 1A). Monocytes treated with CCL2 underwent morphological changes, forming membrane projections or large protrusions from the cell body where actin and tubulin were colocalized (Fig. 1A), characteristic of a polarized migratory phenotype (43–45). Buprenorphine treatment had no effect on monocytes, as evidenced by similar actin and tubulin staining as BSA-treated cells. However, in cells treated with buprenorphine and CCL2, membrane projections were almost completely abolished by cotreatment of cells with CCL2 plus buprenorphine (B) quantification of cells that have cell projections per total number of cells, n = 4 independent experiments. Significance was determined using a two-tailed paired Student t test. **p < 0.01, ***p < 0.005.
Buprenorphine reduces CCL2-mediated monocyte chemotaxis

An important function of monocytes is to migrate from the peripheral vasculature to tissue sites of inflammation in response to chemokines, a process termed chemotaxis (53, 54). To determine the effects of buprenorphine on CCL2-induced chemotaxis, CCL2 (200 ng/ml) was added to the lower wells of a microchemotaxis chamber, and $3 \times 10^5$ human monocytes from five independent donors were added to the upper wells of a chamber. Monocytes were either added directly to the upper wells or pretreated prior to their addition to the chamber. Monocyte migration across a polycarbonate membrane after 25 min at 37˚C with 5% CO$_2$ was quantified.

As shown in the first two columns of Fig. 2, CCL2 was a strong chemoattractant for untreated monocytes as compared with BSA (diluent control, set to one), in agreement with studies published from our laboratory and others (22, 55, 56). We then tested the response of cells pretreated with CCL2 and buprenorphine for their ability to chemotax to CCL2. Cells were pretreated with CCL2, buprenorphine, or CCL2 plus buprenorphine for 15 min at 37˚C, washed, and then added to the upper wells of a microchemotaxis chamber. Pretreatment of cells with buprenorphine alone had no effect on monocyte chemotaxis in response to CCL2 (Fig. 2, Bup pretreatment). In contrast, pretreating monocytes with CCL2 and buprenorphine significantly reduced the chemotactic response to CCL2 (Fig. 2, CCL2 + Bup pretreatment). However, pretreatment of monocytes with CCL2 alone also significantly decreased CCL2-induced chemotaxis (Fig. 2, CCL2 pretreatment), indicative of receptor desensitization and internalization (57, 58).

Thus, additional experiments were necessary to determine whether the reduced chemotaxis exhibited by monocytes pretreated with CCL2 and buprenorphine was due to CCL2 pretreatment or to a buprenorphine-mediated effect on CCL2-treated cells.

The binding of CCL2 to its receptor, CCR2, has been shown to induce phosphorylation of CCR2, resulting in rapid receptor internalization (59, 60). Chemotaxis inhibition by buprenorphine may result from a specific effect of buprenorphine on the recycling of CCR2 to the surface after CCL2-induced receptor internalization. Thus, we pretreated cells with CCL2 or CCL2 plus buprenorphine for 15 min, washed away the treatment, and incubated cells for an additional 30 min prior to addition to the upper wells of a microchemotaxis chamber to allow for recycling of CCR2 to the cell surface. CCL2 was added to the lower wells of the chamber, and chemotaxis was quantified. We found that chemotaxis of monocytes pretreated with CCL2, as described (Fig. 2, CCL2 pretreatment with wash and 30 min of incubation), was similar to that of untreated monocytes (Fig. 2, second column), suggesting pretreatment with CCL2, followed by a 30-min incubation, allows internalized CCR2 to return to the cell surface and mediate chemotaxis. However, with buprenorphine plus CCL2 pretreatment, even after 30 min of additional incubation in media alone, there was a significant decrease in monocyte chemotaxis in response to CCL2 (Fig. 2, CCL2 + Bup pretreatment with wash and 30 min of incubation). These data indicate that exposure to buprenorphine and CCL2 may decrease subsequent monocyte migration to CCL2 by limiting the recycling of CCR2 to the plasma membrane.

CCL2 reduces the surface expression of CCR2, and buprenorphine delays its recycling to the cell membrane

To determine whether buprenorphine and CCL2 treatment delayed the recycling of CCR2 to the monocyte surface, we performed FACS analyses. Monocytes were treated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine, and CCR2 surface expression was quantified by FACS analysis. Fold changes in CCR2 mean fluorescent intensity for all treatments were compared with...
BSA, which was set to one. CCR2 was significantly reduced from the cell surface after treatment with CCL2 for 5 min, as shown from one representative donor (Fig. 3A). Similar results were obtained from nine independent donors (Fig. 3B), as also reported by others (59–61). CCR2 was also internalized after CCL2 treatment for 15 min (data not shown), but maximal internalization occurred after 5 min. Monocytes treated with buprenorphine alone for 5 min had no change in surface CCR2 expression when compared with BSA.

**FIGURE 3.** Buprenorphine delays the recycling of CCR2, the CCL2 receptor, to the cell membrane after CCL2-induced receptor internalization. The surface expression of CCR2 on monocytes was analyzed by flow cytometry after treatment with BSA, CCL2 (200 ng/ml), buprenorphine (20 nM), or CCL2 plus buprenorphine. (A) The change in surface CCR2 was determined by FACS analysis after 5 min of treatment. (B) The fold change in the mean fluorescence intensity (MFI) of CCR2 on monocytes from nine different individuals as compared with BSA (set to one) was calculated after subtracting the contribution of the isotype-matched negative control Ab. The fold change in CCR2 after 5 min of CCL2 or CCL2 plus buprenorphine treatment was decreased as compared with BSA. (C and D) The change in surface CCR2 was determined after treatments, followed by washing and 30 min of incubation to allow for receptor recycling. After pretreatment of cells with CCL2 and then washing and incubation for 30 min, the recycling of CCR2 resulted in higher CCR2 surface expression when compared with 5 min of CCL2 treatment only. In contrast, treatment with CCL2 plus buprenorphine followed by washing and incubation for 30 min resulted in reduced recycling of CCR2 to the surface. (E) The fold change in the mean fluorescence intensity (MFI) of CCR2 on monocytes from five different individuals as compared with control treatment with BSA (set to one) was calculated after subtracting the contribution of the isotype-matched negative control Ab. Data are represented as mean ± SEM. Significance was determined using a two-tailed paired Student t test. *p < 0.05, **p < 0.01.
membrane–spanning protein and a member of the Ig superfamily. JAM-A is demonstrated that JAM-A is important in the transmigration of monocytes across the BBB (23, 24, 67). Homophilic interactions between adhesion proteins on monocytes enable diapedesis (73–75). To examine the effect of buprenorphine on the CCL2-mediated phosphorylation of JAM-A in monocytes, we used Western blotting assays.

Monocytes from eight independent donors were incubated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine for 15 and 30 min. Cell lysates were prepared and analyzed by Western blotting with anti–phospho–p38 Ab. Blots were then stripped and reprobed with anti-p38 Ab. Treatment with buprenorphine alone had no effect on p38 phosphorylation in monocytes from a representative donor (Fig. 4A, 5 min of treatment). We found that p38 phosphorylation was increased after CCL2 treatment alone, and buprenorphine reduced CCL2-induced p38 phosphorylation to near baseline levels. Similar results were obtained with monocytes from seven independent donors (Fig. 4B). Because the time point of CCL2-induced p38 phosphorylation was different in each individual experiment, we used the time point (5 or 15 min) of maximal phosphorylation to quantify the levels of p38 phosphorylation normalized to the amount of total p38 for each experiment. Fold changes in normalized p38 phosphorylation were compared with BSA, which was set to one. These data demonstrate that buprenorphine reduces CCL2-induced p38 phosphorylation, which may contribute to the inhibitory effect of buprenorphine on monocyte chemotaxis in response to CCL2.

Buprenorphine reduces CCL2-induced JAM-A phosphorylation in monocytes

Homophilic interactions between adhesion proteins on monocytes and those on the vascular endothelium facilitate the tightly controlled diapedesis of monocytes into tissues. Our laboratory demonstrated that JAM-A is important in the transmigration of monocytes across the BBB (23, 24, 67). JAM-A is a single membrane–spanning protein and a member of the Ig superfamily.

Buprenorphine decreases CCL2-induced p38 MAPK phosphorylation in human monocytes

To determine whether inhibition of CCL2-induced chemotaxis by buprenorphine may be mediated by alterations in CCL2-mediated signaling in monocytes, we examined p38 MAPK phosphorylation in cells treated with buprenorphine and CCL2. p38 has been reported to be important in the migration of many cells, including monocytes (62–65), and its phosphorylation regulates CCL2-induced monocyte chemotaxis (64–66). To determine the effect of buprenorphine on the CCL2-induced phosphorylation of this kinase, monocytes were treated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine for 5 and 15 min. Cell lysates were prepared and analyzed by Western blotting with anti–phospho–p38 Ab. Blots were then stripped and reprobed with anti-p38 Ab. Treatment with buprenorphine alone had no effect on p38 phosphorylation in monocytes from a representative donor (Fig. 4A, 5 min of treatment). We found that p38 phosphorylation was increased after CCL2 treatment alone, and buprenorphine reduced CCL2-induced p38 phosphorylation to near baseline levels. Similar results were obtained with monocytes from seven independent donors (Fig. 4B). Because the time point of CCL2-induced p38 phosphorylation was different in each individual experiment, we used the time point (5 or 15 min) of maximal phosphorylation to quantify by densitometry the levels of p38 phosphorylation normalized to the amount of total p38 for each experiment. Fold changes in normalized p38 phosphorylation were compared with BSA, which was set to one. These data demonstrate that buprenorphine reduces CCL2-induced p38 phosphorylation, which may contribute to the inhibitory effect of buprenorphine on monocyte chemotaxis in response to CCL2.

Buprenorphine decreases CCL2-induced JAM-A phosphorylation in monocytes

FIGURE 4. Buprenorphine reduces CCL2-induced p38 MAPK phosphorylation. Monocytes from seven different donors were treated with BSA, CCL2 (200 ng/ml), buprenorphine (20 nM), or CCL2 plus buprenorphine. (A) Monocytes were incubated for 5 min, and protein lysates were analyzed by Western blotting using phospho-p38 (anti-p38-P) and total p38 (anti-p38) Abs. In monocytes from one representative donor, CCL2 induced p38 phosphorylation. Treatment with CCL2 + buprenorphine resulted in decreased p38 phosphorylation when compared with CCL2 alone. (B) The time point of p38 phosphorylation was inconsistent due to donor variability, so the time point of maximal phosphorylation (5 or 15 min) in each experiment was analyzed. At the time point of maximal phosphorylation for each experiment, densitometric analysis of p38 phosphorylation, normalized to total p38, indicated a significant increase with CCL2 treatment. Buprenorphine decreased CCL2-induced p38 phosphorylation. n = 7 independent experiments. Significance was determined using a two-tailed paired Student t test; *p < 0.05.

It is expressed at cell–cell junctions of endothelial cells and on monocytes. JAM-A mediates transendothelial migration of leukocytes by regulating the integrity and permeability of cell junctions (68–72). Phosphorylation is a regulatory mechanism to direct the cellular localization and function of these junctional proteins that enable diapedesis (73–75). To examine the effect of buprenorphine on the CCL2-mediated phosphorylation of JAM-A in monocytes, we used Western blotting assays.

Monocytes from eight independent donors were incubated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine for 15 and 30 min. Cell lysates were prepared and analyzed by Western blotting with anti–phospho–JAM-A (serine 284) Ab. Blots were then stripped and reprobed for total JAM-A. The time point (15 or 30 min) of maximal phosphorylation was used to quantify the levels of JAM-A phosphorylation normalized to the amount of total JAM-A for each experiment. Treatment with BSA or buprenorphine alone had no effect on phosphorylation, as shown from a representative donor (Fig. 5A, 15 min of treatment), and in Fig. 5B from eight independent donors. JAM-A was phosphorylated specifically at serine 284 in monocytes with CCL2 treatment. However, treatment of cells with buprenorphine plus CCL2 decreased JAM-A phosphorylation to baseline (Fig. 5A from a representative donor, and Fig. 5B from eight independent donors). Thus, buprenorphine inhibits CCL2-induced phosphorylation of the junctional protein JAM-A, which may contribute to an inhibition of monocyte diapedesis into the CNS in response to CCL2.
Buprenorphine is a partial agonist of the μ-opioid receptor and an antagonist of the κ-opioid receptor. To demonstrate that buprenorphine reduces CCL2-mediated p38 MAPK phosphorylation specifically through opioid receptors and not by off-target effects, we performed a series of experiments using an agonist and an antagonist of the μ-opioid receptor, DAMGO and CTAP, respectively, and an antagonist of the κ-opioid receptor, Nor-BNI. Primary monocytes were either treated with diluent as control, or with CCL2, buprenorphine, CCL2 plus buprenorphine, DAMGO, or CCL2 plus DAMGO, for 5 and 15 min. Cell lysates were prepared and analyzed by Western blotting with anti-phospho-p38 Ab. Blots were then stripped and reprobed with anti-p38 Ab. The time point (5 or 15 min) of maximal phosphorylation was used to quantify the levels of p38 phosphorylation normalized to the amount of total p38 for each condition. As shown in Fig. 4, p38 phosphorylation was increased after CCL2 treatment and buprenorphine reduced CCL2-mediated p38 phosphorylation to baseline levels (Fig. 6A from a representative donor, and Fig. 6B from seven independent donors). DAMGO inhibited CCL2-mediated phosphorylation of p38 similarly as buprenorphine, that is, it reduced CCL2-mediated phosphorylation (Fig. 6A from a representative donor, and Fig. 6B from seven independent donors). When monocytes from different donors were pretreated with CTAP for 30 min and then exposed to control, CCL2, buprenorphine, or CCL2 plus buprenorphine treatments for 5 or 15 min, we found that CTAP blocked the inhibitory effect of buprenorphine on CCL2-induced p38 phosphorylation (Fig. 6C from a representative donor, and Fig. 6D from six independent donors). Thus, our data demonstrated that the effect of buprenorphine on CCL2 signaling is μ-opioid receptor mediated.

To determine whether buprenorphine is acting through the κ-opioid receptor as well, we treated the monocytes with diluent as control, CCL2, buprenorphine, CCL2 plus buprenorphine, Nor-BNI, or CCL2 plus Nor-BNI, for 5 and 15 min. We used monocytes from different donors than were used for the DAMGO or CTAP experiments due to the large number of cells required for each treatment group. Interestingly, as shown in Fig. 6E–H, the effect of Nor-BNI on CCL2-induced p38 phosphorylation was highly donor dependent. In 60% (6 of 10 independent donors) of the donors, Nor-BNI reduced the p38 phosphorylation induced by CCL2, as did CCL2 plus buprenorphine (Fig. 6E from a representative donor, and Fig. 6F from six independent donors). In contrast, for 40% of the donors (4 of 10 independent donors), Nor-BNI had no effect on the increase of p38 phosphorylation after CCL2 treatment (Fig. 6G from a representative donor, and Fig. 6H from 4 independent donors). These results suggest that the effect of buprenorphine on CCL2 signaling is mediated by κ-opioid receptor in some donors, but not for others, and that κ-opioid receptor expression may be variable on these primary cells.

### Proteomic Identification of Membrane Phosphoproteins on Monocytes that are Regulated by CCL2 and Buprenorphine

A large-scale quantitative proteomics analysis of phosphorylated membrane peptides on monocytes was used to identify additional CCL2-induced phosphorylated proteins, with a focus on proteins whose phosphorylation was inhibited by buprenorphine. This proteomic analysis was performed to identify additional mechanisms by which exposure to buprenorphine and CCL2 may inhibit subsequent monocyte transmigration across the BBB in response to CCL2 in the CNS. Monocytes were treated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine for 15 min. Membranes were isolated and proteins were concentrated by acetone precipitation, followed by trypsin digestion. The membrane peptides from the four different treatment groups were labeled with one form of the stable isotope label iTRAQ, 114, 115, 116, or 117 reagents. After pooling the peptides from all the treatment groups, the iTRAQ-labeled phosphopeptides were enriched three times using a TiO2 column and analyzed by LC-MS/MS. This experiment was repeated with monocytes from three different donors. From these different donors, we identified 210, 361, and 388 proteins, respectively, that were phosphorylated in response to any of the four treatments. The Venn diagram in Fig. 7A shows the number of phosphorylated proteins that were expressed by monocytes from each donor, with the number of proteins that were unique to each donor, as well as the number of proteins that were common to two or all three donors. We focused on our subsequent studies on the 149 proteins that were phosphorylated in monocytes from all three donors (Fig. 7A, gray highlight). IPA of these 149 proteins was used to identify molecular networks and biological functions in categories related to monocyte chemotaxis, and the results were as follows: 29 proteins involved in cellular movement, 20 in immune cell trafficking, and 5 in cell-mediated immune responses (Fig. 7B). These proteins were then examined for differences in phosphorylation in monocytes treated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine. We identified 25 proteins whose phosphorylation was increased by CCL2 when compared with BSA (Fig. 7C). Two phosphoproteins of interest,

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**Inhibition of CCL2-mediated p38 MAPK phosphorylation by buprenorphine is specifically through opioid receptors**

Buprenorphine is a partial agonist of the μ-opioid receptor and an antagonist of the κ-opioid receptor. To demonstrate that buprenorphine decreases CCL2-mediated p38 phosphorylation specifically through opioid receptors and not by off-target effects, we performed a series of experiments using an agonist and an antagonist of the μ-opioid receptor, DAMGO and CTAP, respectively, and an antagonist of the κ-opioid receptor, Nor-BNI. Primary monocytes were either treated with diluent as control, or with CCL2, buprenorphine, CCL2 plus buprenorphine, DAMGO, or CCL2 plus DAMGO, for 5 and 15 min. Cell lysates were prepared and analyzed by Western blotting with anti-phospho-p38 Ab. Blots were then stripped and reprobed with anti-p38 Ab. The time point (5 or 15 min) of maximal phosphorylation was used to quantify the levels of p38 phosphorylation normalized to the amount of total p38 for each condition. As shown in Fig. 4, p38 phosphorylation was increased after CCL2 treatment and buprenorphine reduced CCL2-mediated p38 phosphorylation to baseline levels (Fig. 6A from a representative donor, and Fig. 6B from seven independent donors). DAMGO inhibited CCL2-mediated phosphorylation of p38 similarly as buprenorphine, that is, it reduced CCL2-mediated phosphorylation (Fig. 6A from a representative donor, and Fig. 6B from seven independent donors). When monocytes from different donors were pretreated with CTAP for 30 min and then exposed to control, CCL2, buprenorphine, or CCL2 plus buprenorphine treatments for 5 or 15 min, we found that CTAP blocked the inhibitory effect of buprenorphine on CCL2-induced p38 phosphorylation (Fig. 6C from a representative donor, and Fig. 6D from six independent donors). Thus, our data demonstrated that the effect of buprenorphine on CCL2 signaling is μ-opioid receptor mediated.

To determine whether buprenorphine is acting through the κ-opioid receptor as well, we treated the monocytes with diluent as control, CCL2, buprenorphine, CCL2 plus buprenorphine, Nor-BNI, or CCL2 plus Nor-BNI, for 5 and 15 min. We used monocytes from different donors than were used for the DAMGO or CTAP experiments due to the large number of cells required for each treatment group. Interestingly, as shown in Fig. 6E–H, the effect of Nor-BNI on CCL2-induced p38 phosphorylation was highly donor dependent. In 60% (6 of 10 independent donors) of the donors, Nor-BNI reduced the p38 phosphorylation induced by CCL2, as did CCL2 plus buprenorphine (Fig. 6E from a representative donor, and Fig. 6F from six independent donors). In contrast, for 40% of the donors (4 of 10 independent donors), Nor-BNI had no effect on the increase of p38 phosphorylation after CCL2 treatment (Fig. 6G from a representative donor, and Fig. 6H from 4 independent donors). These results suggest that the effect of buprenorphine on CCL2 signaling is mediated by κ-opioid receptor in some donors, but not for others, and that κ-opioid receptor expression may be variable on these primary cells.

### Proteomic Identification of Membrane Phosphopeptides on Monocytes that are Regulated by CCL2 and Buprenorphine

A large-scale quantitative proteomics analysis of phosphorylated membrane peptides on monocytes was used to identify additional CCL2-induced phosphorylated proteins, with a focus on proteins whose phosphorylation was inhibited by buprenorphine. This proteomic analysis was performed to identify additional mechanisms by which exposure to buprenorphine and CCL2 may inhibit subsequent monocyte transmigration across the BBB in response to CCL2 in the CNS. Monocytes were treated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine for 15 min. Membranes were isolated and proteins were concentrated by acetone precipitation, followed by trypsin digestion. The membrane peptides from the four different treatment groups were labeled with one form of the stable isotope label iTRAQ, 114, 115, 116, or 117 reagents. After pooling the peptides from all the treatment groups, the iTRAQ-labeled phosphopeptides were enriched three times using a TiO2 column and analyzed by LC-MS/MS. This experiment was repeated with monocytes from three different donors. From these different donors, we identified 210, 361, and 388 proteins, respectively, that were phosphorylated in response to any of the four treatments. The Venn diagram in Fig. 7A shows the number of phosphorylated proteins that were expressed by monocytes from each donor, with the number of proteins that were unique to each donor, as well as the number of proteins that were common to two or all three donors. We focused on our subsequent studies on the 149 proteins that were phosphorylated in monocytes from all three donors (Fig. 7A, gray highlight). IPA of these 149 proteins was used to identify molecular networks and biological functions in categories related to monocyte chemotaxis, and the results were as follows: 29 proteins involved in cellular movement, 20 in immune cell trafficking, and 5 in cell-mediated immune responses (Fig. 7B). These proteins were then examined for differences in phosphorylation in monocytes treated with BSA, CCL2, buprenorphine, or CCL2 plus buprenorphine. We identified 25 proteins whose phosphorylation was increased by CCL2 when compared with BSA (Fig. 7C). Two phosphoproteins of interest,
S100-A9 and leukosialin/CD43, exhibited a buprenorphine-mediated decrease in CCL2-induced p38 MAPK phosphorylation as defined in Materials and Methods (Fig. 7C, gray highlight).

S100-A9, or MRP14, is a calcium-binding protein highly expressed in monocytes, and is involved in cytoskeletal-membrane interactions and regulation of cytoskeletal reorganization (76–79). Leukosialin, or CD43, is a sialoglycoprotein found on the cell membrane of all leukocytes and has been implicated in the regulation of CD4+ T cell trafficking and T cell migration and activation (80–82). CD43 is expressed in microglia, with higher expression in reactive cells as compared with resting cells (83). The proteomic results of this study suggest that phosphorylation of S100-A9/CD43 in microglia may regulate cytoskeletal reorganization involved in cellular chemotaxis in response to CCL2 and that buprenorphine reduces
this phosphorylation, therefore limiting CCL2-induced monocyte chemotaxis. We are currently examining the role of these phosphoproteins in monocyte diapedesis across the BBB.

**Discussion**

In HIV infection, the MCP, CCL2, is elevated in the brain tissue and CSF of people with HAND (16–19). CCL2 mediates the transmigration of uninfected and HIV-infected monocytes across the BBB into the CNS, thereby playing a key role in neuroinflammation and HAND (84–86). Despite the effectiveness of antiretroviral therapy in the treatment of HIV infection, the prevalence of cognitive, behavioral, and motor abnormalities has increased as infected individuals live longer, and >40–70% of the HIV-infected population exhibit HAND (3–5).

Injection drug use is responsible for 16% of all new HIV infections in the United States (87), and several studies showed that drug abuse increased the severity of cognitive dysfunction in HIV-infected people (88–90). Opiate drug abusers with HIV often have increased neuroinflammation and neuronal damage that may result in accelerated progression of HAND (25–27). As HIV-infected people who abuse drugs live longer, these comorbidities remain a critical public health issue.

Buprenorphine and methadone are the main therapeutics prescribed for people dependent on opiates. Buprenorphine has a different mechanism of action than methadone, indicating that buprenorphine may be a better therapeutic for opioid addiction, particularly in HIV-infected people. Buprenorphine is a partial agonist of the μ-opioid receptor and antagonist of the κ-opioid.
We used DAMGO and CTAP, an agonist and an antagonist of the opioid receptor dependent and not off target. To demonstrate this, the chemotactic response of human primary monocytes in the inflammation by limiting the phosphorylation of JAM-A induced by monocytes indicates that buprenorphine may reduce neuroinflammation sites as well as the use of a protein kinase C inhibitor inhibited plasmic domain deletion mutant lacking several phosphorylation residues involved (Thr326 and Ser 355), and for S100-A9, we found two different residues involved (Thr126 and Ser355), and for S100-A9, we showed phosphorylation at Thr113 (Fig. 7C). We propose that with CCL2 treatment, leukosialin and p38 are phosphorylated, and p38 showning the movement of monocytes. Exposure of monocytes to CCL2 plus buprenorphine leads to phosphorylation of S100-A9, resulting in an increase in the movement of monocytes. Exposure of monocytes to CCL2 plus buprenorphine decreases all of these phosphorylation events and consequently decreases subsequent CCL2-induced cellular movement.

The proteomic data in Fig. 7B and 7C were obtained from the 149 phosphoproteins common to all three donors. TiO2 enrichment identified one phosphopeptide of JAM-A (AA 281–290, KVY5SQpSAR) from two donors and is the same site identified by Western blot. Although the trend for phosphorylation at this site was the same as that observed by Western blot, the changes did not reach the threshold of significance usually required for mass spectrometry (see Materials and Methods). There were no JAM-A peptides detected in the flow-through fraction. The lack of sequence coverage suggests that these proteins are low in abundance. In the case of S100-A9, for example, the sequence coverage is 60, 63, and 78% for the three donors, suggesting that it is...
processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. 


