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*J Immunol* 2005; 175:2655-2665;
doi: 10.4049/jimmunol.175.4.2655
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Morphine Withdrawal Contributes to Th Cell Differentiation by Biasing Cells Toward the Th2 Lineage

Jennifer Kelschenbach,* Roderick A. Barke,† and Sabita Roy2*†

The consequences that drug withdrawal has on immune functioning has only recently been appreciated; however, given the wide variety of use and abuse of opiate analgesics, understanding the decrements to immune function that withdrawal from these drugs causes is of crucial importance. In previous work, we have demonstrated that morphine treatment contributes to immunosuppression by polarizing Th cells toward the Th2 lineage. In the current study, it was hypothesized that morphine withdrawal would result in Th2 differentiation and subsequent immune dysfunction. To address this hypothesis, mice were chronically treated with morphine for 72 h followed by a 24-h withdrawal period. It was determined that 24-h morphine withdrawal resulted in a decrease in IFN-γ, the Th1 signature cytokine, whereas the Th2 cytokine, IL-4, was increased. In addition, Western blot and EMSA experiments revealed that morphine withdrawal-induced Th2 differentiation was mediated through the classical Th2 transcription factors Stat-6 and GATA-3. In addition, the consequence of morphine withdrawal in the presence of an immune stimulation was also examined by treating mice in vivo with LPS before morphine withdrawal. Following withdrawal, it was found that the Th1-polarizing cytokine IL-12 was significantly decreased, providing further support for the observation that withdrawal results in Th2 differentiation by possibly impacting the generation of an appropriate innate immune response which directs subsequent adaptive Th1/Th2 responses. The Journal of Immunology, 2005, 175: 2655–2665.

Clinical and anecdotal evidence provides support for the idea that stressful life experiences may result in impaired immune function and therefore may render an individual more susceptible to infections. This idea of stress-induced immunosuppression can be broadened to include such stresses that are experienced as a result of drug withdrawal, so-called withdrawal-induced immunosuppression. Stress in all of its many forms is considered a huge risk factor for disease, therefore it is paramount to understand the immunological consequences of stress and the mechanisms by which immunosuppression is manifested. It has been documented by the work of many groups that stress significantly alters many aspects of the immune system, at both the adaptive and innate levels (1–3). Our own laboratory has investigated both the effects of stress and chronic morphine treatment on lymphocyte function. Specifically, it was demonstrated that restraint stress increased lymphocyte apoptosis and this loss was not abolished following adrenalectomy in mice (4). In addition, our laboratory has reported that chronic morphine treatment results in the polarization of naive Th cells toward a Th2 effector population (5). The effects of stress and chronic drug exposure on immune function is well reported; however, there are very few investigations into the effects that drug withdrawal has on immune functioning.

The stress associated with drug withdrawal contains both psychological and physical elements, therefore a careful examination of this process and the consequences it imparts on the immune system is warranted. One study examining the effects of cocaine withdrawal on immune functioning found that proliferative responses of peripheral blood lymphocytes to Con A were significantly reduced from 2 h and up to 6 days during cocaine withdrawal. Plasma corticosterone concentrations were significantly elevated for up to 24 h after cocaine cessation, but returned to baseline levels at 2 days of withdrawal. Additionally, the suppressive effects of withdrawal were abolished in animals undergoing adrenalectomy or those administered the glucocorticoid antagonist RU-486, indicating that the effects of withdrawal on cellular immunity were mediated by a glucocorticoid pathway (6). However, it is important to note that deficits in lymphocyte proliferation were seen up to 6 days of withdrawal despite the return of corticosterone to basal levels. This may suggest that the stress associated with drug withdrawal may activate additional pathways that effect immune function. Two reports from Rahim et al. (7, 8) have examined the effects that morphine withdrawal has on immune functioning in mice. Their first study compared the consequences to immune function using both abrupt and precipitated morphine withdrawal, and it was demonstrated that initiation of withdrawal in dependent animals by either method resulted in significant immunosuppression between 24 and 48 h after withdrawal. However, the period of onset and recovery differed between the two modes of withdrawal, with abrupt withdrawal resulting in an early decline followed by a protracted deficit, whereas precipitated withdrawal produced an initial potentiation followed by a decline that recovered within 72 h (7). These findings are interesting because it suggests that the mode of withdrawal also has an effect on the observed immune deficits, suggesting that abrupt withdrawal is more detrimental to immune status, which could possibly be due to the effects of endogenous opiates that may become mobilized during periods of withdrawal. This group of authors postulate that the observed dysfunction was a result of impaired macrophage function, as evidenced by the findings that macrophages obtained from withdrawn spleens displayed reduced expression of the costimulatory molecule B7.2 and had depressed cytokine production (8). This report adds new understanding to the field but cannot explain...
other observed deficits following morphine withdrawal, such as impaired cytotoxic T cell activities (2) and decreased T cell proliferative responses to PHA (9). Additionally, other reports have demonstrated no significant alterations to macrophage function as measured by proliferation and TNF-α production (10). Furthermore, it is difficult to comprehend that all of the dysfunctions to immune operation observed following drug withdrawal are triggered by a deficit to a single component. Drug withdrawal most likely causes a deficit in several immune parameters, and it is the culmination of deficits that results in suppressed immune function. Therefore, it is the goal of this study to focus on the effects of drug withdrawal on Th cell alterations and to explore the underlying mechanisms that result in the observed T cell modifications, with specific focus aimed at examining the classical signaling components involved in Th2 differentiation, specifically Stat-6 and GATA-3.

Materials and Methods

Animals

Six- to 8-wk-old CB6F1/J mice (BALB/cJ female × C57BL/6 male) or B6129SF2 male mice were used in the experiments described within. Animals were housed four animals per cage under controlled conditions of temperature and lighting (12-h light/dark) and given free access to standard food and tap water. All animals were allowed to acclimate to their environment for at least 7 days before any experimental manipulations. All withdrawal protocols were conducted as described in detail below. Sacrifice was performed by carbon dioxide asphyxiation, spleen tissue was harvested aseptically, and blood was collected by either cardiac puncture or retro-orbital bleed. Discomfort, distress, and injury to the animals were minimized. The Institutional Animal Care and Use Committee at the University of Minnesota have approved all protocols in use, and all procedures are in agreement with the guidelines set forth by the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Withdrawal model

Mice were subjected to a well-established model for both generating morphine dependence and producing withdrawal (11). Animals were anesthetized by injection (i.p.) of 300 μl of ketamine and xylazine diluted 1/8 in saline (v/v), followed by implantation with the appropriate pellets (kindly provided by the National Institute on Drug Abuse, Research Triangle Park, NC) depending on the experiment. The implantation procedure consists of making a small incision on the dorsal side of the animal and inserting a 75-mg pellet (placebo or morphine) into the s.c. space created by the incision. Pellets were wrapped with nylon mesh and secured with surgical thread to facilitate easy removal. The incision was closed with the use of stainless steel wound clips. Following the morphine exposure period (72 h), the pellets were removed by making another small incision behind where the pellet was placed; the incision was once again closed with a wound clip. Removal of the pellets initiated spontaneous withdrawal in these animals and it is a widely used and accepted model for generating withdrawal (7). Withdrawal symptoms including, diarrhea, wet dog shakes, tremors, lack of grooming, increased agitation, and up to a 5% reduction in body weight occurred in morphine-withdrawn mice. Some of the experimental groups required implantation of two pellets; the second 25-mg pellet (naltrexone or placebo) was implanted with the first and removed at the time of withdrawal. The morphine withdrawal period consisted of 24 h of spontaneous withdrawal, and at the conclusion of all procedures animals were returned to their home cages, separated by experimental groups, and not housed more than four animals per cage. Following the withdrawal period, animals were sacrificed by CO2 asphyxiation and spleens were harvested as described below. Before sacrifice, blood was collected via the retro-orbital plexus or cardiac puncture.

Tissue culture

Spleens were removed aseptically and suspensions prepared by forcing the tissue through a tissue sieve with a sterile syringe plunger. The cell suspensions were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% newborn calf serum and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were plated at a concentration of 2 × 106 cells/ml, which replicate on 24-well culture plates. Cells were then stimulated with Con A (Sigma-Aldrich) at 5 μg/ml (a concentration determined to have significant stimulatory effect) and incubated overnight at 37°C in 5% CO₂.

ELISAs

Quantikine ELISA kits were obtained (R&D Systems) and assays were run according to the manufacturer’s directions. Briefly, 50–100 μl of sample supernatant or serum was assayed in triplicate per experimental condition. Following the incubation period, plates were washed and 100 μl of detection Ab conjugated to HRP was added to the wells. Finally, 100 μl of substrate solution was added and absorbance was read at 450 nm using a standard plate reader (Packard SpectraCount Microplate Photometer). OD measurements for the standards were used to generate a standard curve, and the concentration of the particular cytokine in each of the samples was extrapolated from this standard curve.

Cytometric bead array (CBA) assay

Mouse Th1/Th2 cytokine CBA assays (BD Biosciences) were performed to confirm data that was generated using traditional ELISA and to provide further support that morphine withdrawal results in Th2 differentiation. This multiplexing system allows for the simultaneous detection of many cytokines from a single sample, specifically this kit measures TNF-α, IFN-γ, IL-4, IL-5, and IL-10. The assay was run according to the manufacturer’s instructions. Briefly, 10 μl/test of each cytokine’s capture bead was added to a well together with a cytokine mixed bead suspension. Then 50 μl of test sample supernatant and 50 μl of PE detection reagent, followed by a 2-h incubation at room temperature. Following the incubation period, samples were washed, centrifuged, and resuspended with wash buffer. Each assay tube was then analyzed using a BD FACSCalibur flow cytometer (BD Immunocytometry Systems). WinMDI software (J. Trotter, The Scripps Research Institute, La Jolla, CA; http://facs.scripps.edu/software.html) was used to analyze and transform PE intensity values for each sample into concentration values (picograms per milliliter). Each sample was run in duplicate and the mean concentrations were expressed as percentage change vs control.

RT-PCR

RT-PCR experiments were used to assess mRNA levels of both cytokines and transcription factors, and were performed as described previously by our laboratory (4). Briefly, total RNA was extracted from splenocytes using an RNasy Mini kit (Qiagen), a system which utilizes spin columns to extract and purify RNA from cells. Before RT-PCR, RNA samples were treated with DNase I (Invitrogen Life Technologies) according to the manufacturer’s instructions. To ensure quality of RNA samples 260/280 UV absorbance readings were performed, and the 260/280 ratios were in range for pure RNA. Total RNA (1 μg) was then reverse transcribed to synthesize first-strand cDNA (45°C, 45 min) using random hexamers (2.5 μM). Moloney murine leukemia virus reverse transcriptase (2.5 U), and 1 mM each of dATP, dCTP, dGTP, and dTTP for a final reaction volume of 40 μl (Applied Biosystems). After first-strand synthesis, the reaction mixture was then heated at 95°C for 5 min to inactivate reverse transcriptase. Amplification steps were performed using upstream and downstream primers specific for mouse IFN-γ (BD Clontech), IL-4 (BD Clontech), GATA-3 (IDT), T-bet (Oligos, Etc.), and β2-microglobulin (BD Clontech). The primer sequences are as follows: IFN-γ, sense 5'TGC ATC TGT GTC GTG CGT TTC TTC TCT ATC GC-3', antisense 5'TGG TGC GGT TGG ACC TCA AAC TTC GC-3'; IL-4, sense 5'CCA AGT ATG GAT CCT GCT CTT CCT TCT CG-3', antisense 5'CGG TGA TGT GGA CTT GGA CTC ATT CAT GGT GC-3'; GATA-3, sense 5'GAA ATC CAG ACC AAC ACC-3', antisense 5'-ACC CTT GCA GAC CAT GC-3'; T-bet sense 5'AAC CAG TAT CCT GCT CCC AGC-3', antisense 5'TGG CTC TGG AAC GAG GAT AGC-3'; and β2-microglobulin, sense 5'ATG GCC CCT CCG GTG ACC CAA-3', antisense 5'ACG TCA TGC TGG ACC CAA-3'. PCR buffer containing 2 mM MgCl₂, 0.1 μM primer, and 2.5 U AmpliTaq DNA polymerase was prepared and −5 μl of the first-strand cDNA reaction mixture was added for a final volume of 50 μl. PCR conditions consisted of 35 cycles of 94°C for 45 s (denaturation), 60°C for 45 s (annealing), and 72°C for 45 s (extension), followed by a final extension at 72°C for 15 min. Minor adjustments to the PCR conditions were made depending on the primer used. Internal controls included both a negative control (containing only PCR mix) and a control lacking reverse transcriptase utilized at the point of cDNA synthesis. PCR products were separated using 1.5% agarose gels and visualized by ethidium bromide staining.

3 Abbreviation used in this paper: CBA, cytometric bead assay.
Western blotting
Protein levels of GATA-3 were determined using Western blot techniques. Nuclear protein extracts were prepared using the Cellytic Nuclear Extraction kit (Sigma-Aldrich). Briefly, cells were resuspended in lysis buffer containing DTT and protease inhibitors. Cells were then homogenized and centrifuged to separate cytoplasmic and nuclear protein. Proteins were then separated by SDS-PAGE using 10% gels, and the gels were then transferred to nitrocellulose membranes using a semidry transfer procedure. Membranes were blocked using Superblock Starting Buffer (Pierce) and probed with a 1/500 dilution of a GATA-3 mouse monoclonal primary Ab (Santa Cruz Biotechnology). Membranes were washed and probed with a 1/2500 dilution of a sheep × mouse secondary Ab conjugated to HRP (Amersham Biosciences). Membrane blots were then exposed to ECL detection reagents (SuperSignal West Pico Chemiluminescent Substrate; Pierce) and visualized using x-ray films. Finally, densitometric analysis was performed to determine fold changes using Stratagene Eagle Eye software.

EMSA
Transcription factor interactions with DNA response elements were assessed using EMSAs as described previously by our laboratory (12). Briefly, nuclear extracts were prepared as described above, Stat-4, Stat-5/6, and GATA consensus oligonucleotides were purchased (Santa Cruz Biotechnology) and end-labeled with 125I according to the manufacturer’s instructions (Promega). Approximately 10 μg of nuclear extracts were incubated with 0.5 ng of labeled probe in binding buffer. DNA-protein complexes were resolved using un-denaturing acrylamide gels. Gels were then dried and visualized by either autoradiography or phosphorimaging techniques.

Corticosterone RIA
Plasma concentrations of corticosterone were assessed using a 125I-labeled double Ab RIA (ICN Biochemicals) according to the manufacturer’s instructions. Concentrations were expressed as nanograms per milliliter.

Morphine RIA
Serum concentrations of morphine were assessed using a 125I competitive immunoassay (Coat-A-Count kit; DPC) according to the manufacturer’s instructions. Concentrations were expressed as nanograms per milliliter.

Withdrawal and infection model
To investigate the consequences of morphine withdrawal in the presence of an in vivo immune stimulation, LPS challenge injections were administered at the time of withdrawal. The model consists of a 72-h implantation of either a placebo or morphine pellet followed by removal initiating withdrawal as described above. However, at the start of withdrawal, 25 μg of LPS (from Escherichia coli O55:B5; Sigma-Aldrich) diluted in saline was injected i.p. Following injections, mice were returned to their home cages where they underwent withdrawal for 24 h. Following the withdrawal period, animals were sacrificed by CO2 asphyxiation and spleens were harvested as described above. Before sacrifice blood was collected via the retro-orbital plexus or cardiac puncture.

Statistics
Each cytokine supernatant protein concentration was expressed as picograms per milliliter ± SEM, and comparisons between group means were assessed using an unpaired Student’s t test. Corticosterone concentration means ± SEM will be plotted according to group and differences assessed using an unpaired t test. Significance will be set at p < 0.05.

Results
Morphine withdrawal results in increased protein synthesis of the Th2 signature cytokine IL-4
The effect of morphine withdrawal on Th cell differentiation was assessed using ELISA and CBA assays. Mice were subjected to 72 h of chronic morphine treatment followed by 24 h of withdrawal. Following the withdrawal period, splenocytes were prepared and stimulated ex vivo with Con A (5 μg/ml) overnight. Cell supernatants were assayed for both IFN-γ (signature Th1 cytokine) and IL-4 (Th2 signature cytokine) using ELISA and CBA assays. It was determined that morphine withdrawal resulted in Th2 differentiation, as evidenced by a significant increase in IL-4 cytokine protein levels following withdrawal compared with placebo withdrawal groups (Fig. 1, A and B). Interestingly, a significant decrease in IFN-γ cytokine protein levels was only observed from splenocyte populations that did not receive Con A stimulation, whereas Con A-stimulated splenocytes displayed similar levels of IFN-γ (Fig. 2A). This discrepancy can be attributed to several factors, such as Con A stimulation resulted in IFN-γ production from naïve Th cells, other cell types, or a ceiling effect was attained. However, given the IL-4 results, we are confident that morphine withdrawal resulted in Th2 differentiation.

To further confirm the ELISA results, CBA analysis was also performed on the splenocyte supernatant collected from morphine- and placebo-withdrawn mice. CBA analysis confirmed what was observed utilizing the ELISA. Specifically, morphine withdrawal resulted in a significant increase in IL-4 protein levels from both stimulated and nonstimulated splenocytes as compared with placebo controls. Fig. 1B is dot plot representations depicting the PE intensity, which correlates with cytokine concentration, of samples taken from Con A-stimulated placebo- and morphine-withdrawn splenocytes, respectively. The CBA analysis also confirmed what was observed with the ELISA with regard to the changes in IFN-γ protein levels. Specifically, morphine withdrawal resulted in a significant decrease in IFN-γ protein levels from only nonstimulated splenocytes as compared with placebo controls. Once again there were no significant differences between IFN-γ levels from the Con A-stimulated splenocytes harvested from morphine- or placebo-withdrawn animals. Fig. 2B is also dot plot representations depicting PE intensity of samples taken from nonstimulated placebo and morphine-withdrawn splenocytes, respectively. Finally, through the use of two methods, which detect Th1/Th2 cytokine levels, it can be suggested that morphine withdrawal results in Th2 polarization, and these changes in cytokines are attributable solely to withdrawal because serum morphine concentrations at this time point were negligible (data not shown).

Morphine withdrawal results in increased message levels of the Th2 signature cytokine IL-4
The effects of morphine withdrawal on Th cell polarization was also assessed using RT-PCR experiments to examine the effects on both cytokine and transcription factor mRNA levels. The cytokines that were examined include the conventional Th1 cytokine IFN-γ and the classical Th2 cytokine IL-4. The transcription factors T-bet and GATA-3 serve as molecular switches, which drive the process of Th cell differentiation toward either Th1 or Th2 effector populations, respectively; therefore, the message levels of these two transcription factors were also examined. Splenocytes isolated from both placebo- and morphine-withdrawn mice were stimulated overnight with Con A (5 μg/ml), harvested for total RNA, and RT-PCR experiments were performed as described above. Experiments revealed that the message levels of the two cytokines were in accordance with the observed protein levels of the same cytokine as assessed by ELISA, that is morphine withdrawal resulted in a significant increase in the IL-4 message following Con A stimulation compared with placebo controls (Fig. 1C), whereas there were no significant changes in IFN-γ levels following stimulation (Fig. 2C). These experiments suggest that morphine withdrawal results in Th2 differentiation as evidenced by an increase in the IL-4 message. The absence of changes to IFN-γ levels can once again be explained by the concept that splenocytes are a mixed cell population and the IFN-γ may have been produced by another cell type (NK cells). Once again, however, it can be implied that morphine withdrawal does impact Th cell differentiation by resulting in Th2 polarization.
To investigate upstream mechanisms by which withdrawal from morphine may be mediating Th2 differentiation, RT-PCR experiments were performed on the Th1/Th2-specific transcription factors T-bet and GATA-3. It was determined that morphine withdrawal did not significantly alter the message levels of either T-bet or GATA-3 as compared with placebo controls (data not shown). These results suggest that withdrawal from morphine does not directly impact the generation at the message level of either of these factors.
crucial transcription factors; however, these experiments do not rule out the possibility that withdrawal may be facilitating factor binding to DNA response elements or promoting transcription factor translocation to the nucleus, which are experiments that will be addressed in the succeeding studies.

Morphine withdrawal promotes GATA-3 translocation to the nuclear compartment

With the goal to further understand the molecular mechanisms which underlie morphine withdrawal-mediated Th2 differentiation, Western blot experiments were performed on splenocytes isolated from both placebo- and morphine-withdrawn animals. Nuclear protein extracts were prepared from the splenocyte cultures and Western blots probing for GATA-3 protein were ran as described above. These experiments revealed that morphine withdrawal increased the levels of GATA-3 protein in the nuclear compartment by ~5-fold (Fig. 3) compared with controls. This increase was seen with both Con A-stimulated and nonstimulated cells (Fig. 3A, lanes 3 and 4), which adds support to our ELISA findings (Fig. 1A) which demonstrated that both stimulated and nonstimulated cells displayed significant increases in IL-4 as compared with placebo controls. Interestingly, animal receiving the opiate antagonist naloxone alone (Fig. 3A, lanes 5 and 6) displayed decreased levels of GATA-3 as compared with placebo controls (Fig. 3A, lanes 1 and 2). These findings are in agreement with studies published by Sacerdote et al. (13) in which they reported that naloxone treatment results in Th1 differentiation, and therefore it can be speculated that GATA-3 levels would be decreased. In addition, the
groups that received naltrexone at the time of withdrawal (Fig. 3A, lanes 7 and 8) possessed GATA-3 levels that were similar to control levels, possibly suggesting that at the time of withdrawal endogenous opiates may be mobilized that contribute to Th2 polarization, and this effect can be blocked by administration of a nonselective opiate antagonist. Further studies are ongoing which address the contribution of endogenous opiates and the opiate receptors to this process of morphine withdrawal-mediated Th2 polarization. Nevertheless, it can be inferred from the current experiments that withdrawal from morphine contributes to Th2 differentiation by promoting GATA-3 translocation to the nuclear compartment.

Morphine withdrawal results in enhanced DNA response element binding of the transcription factors Stat-5/6 and GATA

As mentioned previously, the transcription factors Stat-6 and GATA-3 are important intermediates in the signal transduction pathways leading to Th2 differentiation. EMSAs were used to examine the impact that withdrawal from morphine has transcription factor binding to Stat and GATA consensus oligonucleotide sequences. Nuclear proteins extracts were prepared and EMSAs were conducted using \(^{32}P\)-labeled probes as described above. The gel shift experiments revealed that morphine withdrawal enhanced binding of Stat-5/6 (Fig. 4B) and GATA (Fig. 4C) to consensus sequences as compared with placebo controls. Once again, naltrexone alone groups displayed binding that was similar to control levels. Interestingly, naltrexone alone groups displayed enhanced binding compared with controls, once again in agreement with previous work, which demonstrated that naloxone promotes Th1 differentiation (13). Taken together the EMSA studies provide further support that withdrawal from morphine polarizes Th cells toward the Th2 lineage and that this process is mediated by the classical Th2 transcription factors Stat-6 and GATA.

**FIGURE 3.** Morphine withdrawal results in increased GATA-3 translocation to the nuclear compartment. Splenocytes were harvested from placebo, morphine-withdrawn, naltrexone alone, and morphine-withdrawn + naltrexone CB6F1/J mice and cultured overnight with or without Con A (5 \(\mu\)g/ml). Nuclear extracts were prepared and separated by SDS-PAGE. Blots were then probed with a mouse × rodent primary mAb, followed by a probing with a sheep × mouse secondary Ab, and visualized using ECL detection reagents (A, upper panel). Blots were then stripped and probed with an \(\alpha\)-tubulin primary Ab to ensure for equivalence of protein levels (A, lower panel). Densitometry was then performed on the films to determine fold changes in GATA-3 levels over \(\alpha\)-tubulin levels (B). Lane 1, placebo withdrawal; lane 2, placebo withdrawal + Con A; lane 3, morphine withdrawal; lane 4, morphine withdrawal + Con A; lane 5, naltrexone alone; lane 6, naltrexone alone + Con A; lane 7, morphine withdrawal + naltrexone; and lane 8 = morphine withdrawal + naltrexone + Con A.
cell differentiation, given that IL-12 is the major Th1-polarizing support to our findings that morphine withdrawal contributes to Th1-polarizing cytokines, add further compared with placebo controls receiving LPS (Fig. 5). Stimulation there was a significant decrease in IL-12p70 levels was demonstrated that following withdrawal in the context of LPS stimulation there was a significant decrease in IL-12p70 levels compared with placebo controls (Fig. 5C). There was a trend for a decrease in IFN-γ levels in cells treated ex vivo with LPS; however, this difference was not significant. Strikingly, Con A stimulation ex vivo resulted in a stimulation of both placebo- and morphine-withdrawn cells, therefore resulting in no significant difference between the groups. This result was quite consistent and it suggests that Con A stimulation is stimulating Th0 cells in both cell populations, resulting in increased levels of IFN-γ and no significant differences between the groups. In contrast, morphine-withdrawn cell cultures stimulated ex vivo with Con A displayed increased protein (Fig. 5D) and message levels of IL-4 (data not shown) compared with placebo controls. This finding suggests that the morphine withdrawn cells have undergone Th2 polarization and once stimulated produce elevated levels of IL-4 compared with placebo control cultures.

Message levels of the transcription factors T-bet and GATA-3, which are involved in the differentiation into either Th1 or Th2 effector populations, respectively, were also assessed following in vivo LPS stimulation. It was demonstrated that morphine withdrawal in the presence of LPS stimulation increased mRNA levels of GATA-3 while reducing the levels of T-bet (Fig. 6), once again supporting the findings that morphine withdrawal contributes to Th2 polarization in the context of an LPS infection.

**Morphine withdrawal-mediated Th2 differentiation is an opiate-mediated event and is not associated with corticosterone levels**

When examining withdrawal in the context of a stress response the contribution of the hypothalamic-pituitary-adrenal axis and glucocorticoids, members of the classical stress pathway, need to be considered. To this end, serum concentrations of corticosterone were measured using a double Ab 125I-labeled RIA as described above. It was revealed that following morphine withdrawal there were no significant elevations in corticosterone levels as compared with placebo controls (Fig. 7). These results contradict the findings of others, which have demonstrated that 24 h of morphine withdrawal results in significant elevations of corticosterone levels as compared with placebo controls (Fig. 7). These results indicate that corticosterone does not contribute to this process.

**Discussion**

In summary, the findings presented clearly demonstrate that morphine withdrawal contributes to Th2 polarization. The studies presented using both ELISA and CBA assays demonstrate that withdrawal from morphine results in increases in IL-4 protein levels (Fig. 1), which is a clear indicator of Th2 differentiation given the understanding that only fully differentiated or differentiating Th2 cells produce IL-4. The results obtained for IFN-γ levels were somewhat troubling, because it was expected that these levels would be decreased in morphine-withdrawal groups. However, as mentioned previously other cell types in the mixed population of splenocytes could have attributed to the observed levels of IFN-γ, or simply that the detection system used was not able to reveal any significant changes in IFN-γ following Con A stimulation. The

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**FIGURE 4.** Morphine withdrawal results in enhanced DNA response element binding of Stat-5/6 and GATA. Splenocytes were harvested from placebo, morphine-withdrawn, naltrexone alone, and morphine withdrawn + naltrexone CB6F1/J mice and cultured overnight with Con A (5 μg/ml). Nuclear extracts were prepared and incubated with the following 32P-labeled consensus sequences, Stat-4 (A), Stat-5/6 (B), and GATA (C). DNA-protein complexes were run on non-denaturing acrylamide gels and visualized by autoradiography. Lane 1, placebo withdrawal + Con A; lane 2, morphine withdrawal + Con A; lane 3, naltrexone alone + Con A; and lane 4, morphine withdrawal + naltrexone + Con A.

**Morphine withdrawal in the presence of an in vivo LPS stimulation results in Th2 polarization**

In vivo LPS stimulation was performed at the time of withdrawal to examine the effects that withdrawal has on immune function in the context of an infection. Once again, mice were subjected to 72 h of chronic morphine treatment followed by 24 h of spontaneous withdrawal. However, at the start of withdrawal LPS was administered to both placebo control and morphine withdrawal animals, whereas saline was administered to the other half of the experimental animals. Following the 24-h withdrawal period, mice were bled via cardiac puncture and spleens were harvested. Plasma levels of both IL-12p70 and IFN-γ were assessed using ELISA. It was demonstrated that following withdrawal in the context of LPS stimulation there was a significant decrease in IL-12p70 levels compared with placebo controls receiving LPS (Fig. 5A). Decreased levels of IL-12, the Th1-polarizing cytokine, add further support to our findings that morphine withdrawal contributes to Th cell differentiation, given that IL-12 is the major Th1-polarizing cytokine. In addition, plasma levels of IFN-γ were assessed and once again it was found that morphine withdrawal resulted in significant decreases in this Th1 cytokine compared with placebo controls (Fig. 5B).

Splenocyte preparations were also generated from these same animals and stimulated overnight with either LPS (5 μg/ml) or Con A (5 μg/ml). Supernatants from these cell cultures were assayed for protein levels of both IFN-γ and IL-4, and cell fractions were used to isolate RNA and assess message levels of these same cytokines. It was found that morphine withdrawal once again resulted in a significant decrease in IFN-γ protein levels and this decrease was most evident in the cells receiving no ex vivo treatment (Fig. 5C). There was a trend for a decrease in IFN-γ levels in cells treated ex vivo with LPS; however, this difference was not significant. Strikingly, Con A stimulation ex vivo resulted in a stimulation of both placebo- and morphine-withdrawn cells, therefore resulting in no significant difference between the groups. This result was quite consistent and it suggests that Con A stimulation is stimulating Th0 cells in both cell populations, resulting in increased levels of IFN-γ and no significant differences between the groups. In contrast, morphine-withdrawn cell cultures stimulated ex vivo with Con A displayed increased protein (Fig. 5D) and message levels of IL-4 (data not shown) compared with placebo controls. This finding suggests that the morphine withdrawn cells have undergone Th2 polarization and once stimulated produce elevated levels of IL-4 compared with placebo control cultures.

Message levels of the transcription factors T-bet and GATA-3, which are involved in the differentiation into either Th1 or Th2 effector populations, respectively, were also assessed following in vivo LPS stimulation. It was demonstrated that morphine withdrawal in the presence of LPS stimulation increased mRNA levels of GATA-3 while reducing the levels of T-bet (Fig. 6), once again supporting the findings that morphine withdrawal contributes to Th2 polarization in the context of an LPS infection.
observation that IFN-γ levels did not increase following Con A stimulation of placebo-withdrawn cells lend support to the possibility that a ceiling effect had been reached with the detection systems used. However, given the IL-4 results, we are confident that morphine withdrawal resulted in Th2 differentiation, because only fully differentiated or differentiated Th2 cells produce IL-4.

FIGURE 5. Morphine withdrawal in the presence of LPS results in decreased IL-12p70 and IFN-γ production in both the serum and supernatant. Serum was collected from B6129SF2 mice via cardiac puncture or retro-orbital bleed. Serum levels of IL-12p70 (A) and IFN-γ (B) were assessed using ELISA. Splenocytes were also collected and stimulated overnight with or without Con A (5 μg/ml). IFN-γ levels assessed in samples not receiving Con A (C) and IL-4 levels assessed in samples stimulated with Con A (D) are presented. Each experimental group contained at least six mice, and the data presented are representative of at least two experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 

"MORPHINE WITHDRAWAL AND Th2 DIFFERENTIATION"
The results obtained from RT-PCR experiments confirm what was observed at the protein level, i.e., there were increased levels of IL-4 message following morphine withdrawal, and no significant changes in IFN-γ mRNA. Despite the apparent contradiction with the protein and message level data, the results obtained following LPS infection provide convincing evidence that morphine withdrawal promotes Th2 polarization.

Studies revealed that withdrawal resulted in a nearly 5-fold increase in the crucial Th2 transcription factor GATA-3 in the nuclear compartment (Fig. 3). It has been demonstrated by others that GATA-3 is necessary and sufficient to turn on a Th2 differentiation program (15), and it is demonstrated here that morphine withdrawal facilitates this process, thus adding further support to the observed increases in IL-4 protein. EMSA experiments were also performed to further examine how withdrawal promotes Th2 differentiation at the level of the signaling pathway. It was demonstrated that withdrawal from morphine resulted in enhanced DNA response element binding of the Th2 transcription factors Stat-5/6 and GATA (Fig. 4). In contrast, the Th1 transcription factor Stat 4 displayed decreased binding following morphine withdrawal. This finding was interesting given that Con A stimulation resulted in no significant changes between placebo and morphine withdrawn splenocytes (Fig. 2A). Recently, it has been reported that CD40 engagement enhances APC priming of IFN-γ-producing cells independently of IL-12 (16). This report suggests that CD40 ligation utilizes a mechanism distinct from IL-12 and Stat-4 to promote T cell development to IFN-γ-producing cells. Furthermore, it has also recently become clear that induction of IFN-γ appears to be regulated by two distinct pathways, one TCR mediated and the other mediated by IL-12 and Stat-4 (17). It is clear from the in vivo LPS experiments that IL-12-mediated IFN-γ production is inhibited by morphine withdrawal (Fig. 5). However, the in vitro Con A data is somewhat perplexing (Fig. 2), given that we observed decreased Stat-4 binding with no significant changes observed in IFN-γ production. This discrepancy can be explained by the observation that mitogen treatment of Stat-4-deficient cells results in robust generation of IFN-γ (17). Thus, it can be speculated that Con A treatment is bypassing upstream signaling components and stimulating IFN-γ production through stimulation of the p38 MAP kinase pathway without prior involvement of Stat-4. The effects that morphine withdrawal has on Th2 signaling mechanisms cannot be denied, and it can be stated that withdrawal from morphine contributes to the process of Th cell differentiation with a preferential skewing toward Th2 effector populations.

The effect of morphine withdrawal on T cell polarization was also examined in the context of an infection, and it was demonstrated that withdrawal resulted in Th2 differentiation (Figs. 5 and 6). Our laboratory had previously demonstrated that low doses of morphine synergize with LPS, resulting in both increased mortality and a shift in cytokine profile from Th1 to Th2, and it was concluded that the use of morphine as a postsurgical analgesic should be given careful examination due to increased risk for sepsis (18). The same conclusions hold for the current studies due to the observations that withdrawal from morphine also contributes to Th2 polarization in the context of LPS stimulation. The mechanisms by which both morphine withdrawal and chronic morphine treatment result in the same outcome are under active investigation by both our laboratories and others; however, to our knowledge, we are the first group to demonstrate that morphine withdrawal contributes to Th2 differentiation. Furthermore, it is also worthwhile to mention that the model we have used mimics more of a withdrawal paradigm experienced following morphine administered to treat postsurgical pain, as opposed to the type of withdrawal experienced by drug addicts who encounter morphine more frequently and suffer repeated periods of withdrawal. This difference and the consequences to Th cell differentiation as a result of these differences is under active investigation in our laboratory.

Several of the experiments performed in this study were designed to address the fundamental molecular mechanisms that morphine withdrawal corrupts to trigger Th2 polarization. However, many questions still need to be addressed, including what is the major upstream factor that initiates the differentiation program? Several authors have demonstrated that both chronic use of a drug and subsequent withdrawal result in hypothalamic-pituitary-adrenal axis activation and consequent increases in glucocorticoids (6, 14, 19, 20). The immunosuppressive effects of glucocorticoids have been appreciated for nearly two decades, and it seems plausible to conclude that the stress associated with chronic

**FIGURE 6.** Morphine withdrawal in the presence of LPS results in an increase in the GATA-3 message and a decrease in the T-bet message. Total RNA was isolated from splenocytes harvested from placebo + LPS and morphine-withdrawn + LPS B6129SF2 mice and RT-PCR experiments were performed to assess mRNA levels of T-bet and GATA-3. The housekeeping gene β2-microglobulin was used to monitor equivalence of loading.

**FIGURE 7.** Morphine withdrawal-mediated Th2 differentiation is not mediated by corticosterone levels. Blood was collected from experimental CB6F1/J mice by cardiac puncture and/or retro-orbital bleed following the 24-h withdrawal period. Serum concentrations of corticosterone were assessed using a125I-labeled double Ab RIA. Mean concentrations were expressed as nanograms per milliliter ± SEM.
drug taking and/or withdrawal enhances glucocorticoid levels, which contributes to immune dysfunctions. In addition, glucocorticoid (corticosterone) treatment has also been demonstrated to inhibit Th1 and enhance Th2 development (21), once again suggesting that a rise in glucocorticoid levels is a major contributing factor to immunosuppression. In contrast, it has also been demonstrated that the immunosuppressive effects associated with chronic morphine treatment are not entirely dependent on corticosterone levels, specifically deficits in IFN-γ synthesis and macrophage cytokine production are independent of corticosterone levels (22). Furthermore, when examining withdrawal-mediated deficits in immune functioning, no significant changes in corticosterone levels are observed (Fig. 7) or the elevations in corticosterone cannot explain prolonged immune dysregulation (14). Therefore, there are other yet unknown factors that are contributing to this process that may or may not be working in concert with corticosterone to bring about the observed deficits.

Several hypotheses have been put forth regarding the molecular mechanisms by which chronic morphine treatment and/or withdrawal imparts immune failure, including as mentioned that the stress associated with these processes elevates glucocorticoid levels resulting in dysfunction. However, other mechanisms have been proposed which postulate that the drug acts directly on the cells of the immune system to bring about impairments, and others have suggested that endogenous opiates are released, which mediate the observed phenomenon, and finally that Th2 differentiation serves as a default pathway in the case of improper Th1 polarization. In light of the findings presented in the current study, attentions have been focused on the later two proposals in the pursuit of understanding the upstream mechanisms involved in morphine withdrawal-mediated Th2 differentiation.

It has been reported that Th2 differentiation serves as a default pathway to Th1 polarization, and cells are directed toward this pathway when the repressing signal of IL-12 is absent (23). We have reported in the current study that morphine withdrawal results in decreased serum levels of IL-12p70 in the presence of in vivo LPS stimulation (Fig. 5A). Other investigators have also attributed this “default” pathway as the mechanism by which cannabinoids exert Th2 differentiation (24, 25). Therefore, we propose that morphine withdrawal is contributing to Th2 differentiation by affecting the innate immune response and inhibiting the production of IL-12, which therefore results in a defaulting of cells toward the Th2 pathway. Defects in macrophage function following withdrawal have been noted by other investigators (8), which lend support to the current hypothesis.

Differentiation along the Th2 pathway must be maintained and it is hypothesized that the release of endogenous opiates acting on the Th cell (via opioid receptors) is the mechanism by which Th2 polarization is maintained. It is well documented that all three of the classical opioid receptors (μ, κ, and δ) are expressed by cells of the immune system, including Th cells (26–29). In addition, many studies from our laboratory have also demonstrated that certain morphine-related immune impairments are abolished in μ opioid receptor knock out (MORKO) mice, including the loss of stress-induced lymphocyte apoptosis (4) and Th cell differentiation toward the Th2 lineage (5). These results therefore suggest that morphine is acting directly on lymphocytes (via the μ opioid receptor) to bring about the observed deficits. This idea of molecular adaptations to chronic morphine exposure has been appreciated for several years in the CNS. One of the classical indicators of cellular adaptations following chronic morphine exposure is up-regulation of the adenylyl cyclase/cAMP pathway, which opposes the acute inhibition produced by initial exposure. It is then believed that removal of the opiate drug activates the functional up-regulated pathway, resulting in changes to underlying signaling pathways and mechanisms (30). This idea of chronic drug exposure resulting in cellular adaptations has yet to be explored as it pertains to the immune system. Interestingly however, it has been reported that cAMP-elevating agents promote Th2 differentiation as evidenced by increased levels of the Th2 cytokines IL-4 and IL-5 (31). In addition, studies in our laboratory have demonstrated that morphine withdrawal results in increased phosphorylation of CREB, a downstream target of cAMP (our unpublished findings), thus suggesting that adenylyl cyclase superactivation may be involved in the retention of the Th2 phenotype following morphine withdrawal.

The second hypothesis that the stress associated with withdrawal mobilizes endogenous opiates, which facilitates this process of Th2 differentiation is also under active investigation by our laboratory. As mentioned previously, it has been reported that naloxone treatment results in Th1 differentiation, and it was hypothesized that naloxone removed the tonic inhibitory effects of β-endorphin, which may be supporting Th2 polarization (13). In addition, preproenkephalin mRNA and met-enkephalin were present at higher levels in Th2 cell cultures, suggesting that the enkephalins may also contribute to Th2 differentiation (32). Given these findings, investigating the involvement of endogenous opiates in withdrawal-mediated Th2 polarization would be an intriguing avenue to explore. Finally, we are proposing that morphine withdrawal is preventing Th1 differentiation (evidenced by decreases in IL-12p70) and resulting in a biasing toward the Th2 lineage, and maintained expression of this Th2 phenotype may be the result of a mobilization of endogenous opiates which signal via opioid receptors through a withdrawal adapted, cAMP superactivated pathway resulting in modulations to GATA-3, ultimately leading to Th2 differentiation (Fig. 8).

In closing, this study has examined the effects that morphine withdrawal has on Th cell function, demonstrating that withdrawal promotes Th2 differentiation by a Stat-6/GATA-3-mediated mechanism. Finally, examining morphine withdrawal-induced immunosuppression is a worthwhile endeavor given the wide use and abuse of opiates, therefore understanding the mechanisms by which withdrawal renders an individual prone to infection is a meaningful goal.
Acknowledgments
We thank Dr. Kevin Wickman and Maria Roman for use of the B6129SF2 mouse strain and Richard Charboneau for valuable technical support.

Disclosures
The authors have no financial conflict of interest.

References