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Chronic Morphine Treatment Promotes Specific Th2 Cytokine Production by Murine T Cells In Vitro via a Fas/Fas Ligand-Dependent Mechanism

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Improper homeostasis of Th1 and Th2 cell differentiation can promote pathological immune responses such as autoimmunity and asthma. A number of factors govern the development of these cells including TCR ligation, costimulation, death effector expression, and activation-induced cell death (AICD). Although chronic morphine administration has been shown to selectively promote Th2 development in unpurified T cell populations, the direct effects of chronic morphine on Th cell skewing and cytokine production by CD4+ T cells have not been elucidated. We previously showed that morphine enhances Fas death receptor expression in a T cell hybridoma and human PBL. In addition, we have demonstrated a role for Fas, Fas ligand (FasL), and TRAIL in promoting Th2 development via killing of Th1 cells. Therefore, we analyzed whether the ability of morphine to affect Th2 cytokine production was mediated by regulation of Fas, FasL, and TRAIL expression and AICD directly in purified Th cells. We found that morphine significantly promoted IL-4 and IL-13 production but did not alter IL-5 or IFN-γ. Furthermore, morphine enhanced the mRNA expression of Fas, FasL and TRAIL and promoted Fas-mediated AICD of CD4+ T cells. Additionally, blockade of Fas/FasL interaction by anti-FasL inhibited the morphine-induced production of IL-4 and IL-13 and AICD of CD4+ T cells. These results suggest that morphine preferentially enhances Th2 cell differentiation via killing of Th1 cells in a Fas/FasL-dependent manner. The Journal of Immunology, 2005, 175: 4999–5005.

There are two major subsets of Th cells, Th1 and Th2, which arise after antigenic stimulation (1–9). These cells are categorized based upon the cytokines they produce and the induction of cellular or humoral immunity, respectively. Th1 cells secrete IFN-γ and TNF-α to mediate the destruction of intracellular pathogens. Th2 cells secrete IL-4, IL-5, and IL-13 and promote Ab production and the removal of extracellular pathogens. In addition, the two Th cell phenotypes are known to reciprocally inhibit each other. To date, multiple mechanisms have been proposed to govern the development of Th1 vs Th2 cells, including activation through the TCR, costimulatory molecules, the induction of transcription factors, and the external cytokine environment (9). Stimulation through the TCR in the absence of exogenous factors causes low, nonspecific transcription of both IFN-γ and IL-4; however, skewing of Th cell differentiation can be efficiently achieved in vitro by the addition of IL-12 (Th1 conditions) or IL-4 (Th2 conditions).

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Interestingly, corticosterone, neuropeptides, and drugs of abuse, including cocaine and morphine, have been shown to enhance Th cell-dependent Ab responses, increase levels of IL-4, and shift T cell functions to the Th2 type via binding to classical opioid receptors (10, 11). The opioid receptor antagonist naloxone has been shown to enhance Th1-mediated responses, including skin graft rejection, in a murine model (12). In these studies, naloxone increased the production of IL-2 and IFN-γ by splenocytes to promote rejection, indicating that endogenous β-endorphin normally inhibits IFN-γ production. Furthermore, naloxone was shown to induce a shift from type 2 to type 1 cytokine production in mice immunized with keyhole limpet hemocyanin (KLH) (13). Administration of morphine in vitro has also been shown to direct T cells toward Th2 responses. Treatment of total splenocytes with morphine for 4 days in the presence of anti-CD3/anti-CD28 stimulation resulted in decreases in IL-2 and IFN-γ with a concomitant increase in IL-4 production (11). Additionally, chronic morphine treatment of splenocytes caused a decrease in IFN-γ and an increase in IL-4 and IL-5 mRNA levels (14).

Th1 and Th2 cells are also known to have different sensitivities to activation-induced cell death (AICD) (15–17). After TCR ligation, Th1 cells express significantly higher levels of Fas ligand (FasL) than Th2 cells and are known to be highly susceptible to Fas-mediated apoptosis, whereas Th2 cells are resistant (18). The resistance of Th2 cells to AICD can be explained by the observation that Th1 clones, but not Th2 clones, express FasL mRNA in response to activation signals (19). However, this dichotomy of FasL expression in Th1 vs Th2 cells is controversial; one report showed that differentiated murine Th1 and Th2 cells both express FasL and are equally susceptible to Fas-mediated AICD (20).

4 Abbreviations used in this paper: AICD, activation-induced cell death; CBA, cytometric bead array; CT, threshold cycle; ∆∆CT, comparative CT analysis method; DOR, δ opioid receptor; KLH, keyhole limpet hemocyanin; MOR, μ opioid receptor; PKC, protein kinase C; rm, recombinant murine.
We recently reported that TRAIL and FasL are expressed in distinct patterns in both cloned and in vitro differentiated Th1 and Th2 cells upon activation through the TCR (21). TRAIL was found to be exclusively expressed in Th2 cells, whereas FasL was the primary ligand expressed in Th1 cells, indicating that the expression of TRAIL or FasL may be used to classify Th cells. Furthermore, Th2 cells were shown to be more resistant to either TRAIL- or FasL-induced apoptosis than Th1 cells, and blockade of TRAIL or blockade of FasL significantly enhanced IFN-γ and decreased IL-4 production by activated Th cells (21). These results indicate that after TCR ligation, Fas and FasL expression on Th1 cells and TRAIL expression on Th2 cells are involved in the selective apoptosis of Th1 cells by either suicide or Th2-mediated fratricide by binding of TRAIL on the Th2 cells to its receptor DR4 on the Th1 cell (22). We have also shown that morphine induces Fas receptor expression in the T cell hybridoma, A1.1 and in freshly isolated human PBL and primes lymphocytes for FasL-mediated apoptosis in vitro (23).

In this study we examined the ability of morphine to directly promote Th2 differentiation in purified CD4+ T cells via induction of Fas, FasL, and/or TRAIL expression to promote killing of Th1 cells. We found that morphine significantly induced IL-4 and IL-13 production in a FasL-dependent manner. In addition, morphine enhanced the mRNA expression of Fas, FasL, and TRAIL and promoted AICD of CD4+ T cells. These results suggest that in addition to TCR signaling and the cytokine environment, signaling via opioid receptors functions to modulate the outcome of Th cell differentiation via modulation of AICD.

Materials and Methods

**Mice, reagents, and Abs**

Female C57BL/6 mice, 5–6 wk of age, were obtained from Taconic Farms. Mice were maintained in the vivarium of the Jerome H. Holland Laboratory of the American Red Cross under specific-pathogen-free conditions with a sentinel mouse program, and food and water were provided ad libitum. Animals were gender and age matched in each experiment. The animal care and use protocol was approved by the institutional animal care and use committee of the Jerome H. Holland Laboratory of the American Red Cross. Naltrexone hydrochloride and morphine sulfate were purchased from Sigma-Aldrich. Murine anti-CD3ε Ab (clone 2C11) was prepared by Dr. L. Zhang (Jerome H. Holland Laboratory of the American Red Cross, Rockville, MD). Murine anti-CD28 Ab and murine anti-CD95L (clone M3L3) Ab were purchased from BD Pharmingen. Recombinant human IL-2 was purchased from R&D Systems.

**Purification of CD4+ T cells**

C57BL/6 CD4+ T lymphocytes were purified from lymph nodes and spleens using CD4+ T cell high affinity negative selection columns (R&D Systems). Briefly, lymph nodes and spleens were collected and mashed between the frosted ends of two sterile microscope slides. Single-cell suspensions were resuspended in ACK lysis buffer for 5 min at room temperature. Cells were washed twice, then resuspended in fresh wash buffer and applied to columns. Upon incubation at room temperature. Cells were washed twice, then resuspended in fresh, noncoated, 24-well plates on day 4 of culture before being used for all experiments. These conditions are termed neutral conditions, as previously described (21). For morphine studies, increasing concentrations of morphine were added on day 1 of culture in the presence or the absence of naltrexone or anti-Fasl. Cells were incubated as described above, and on day 4 of culture, cytokines, morphine, and/or naltrexone were added. Cells were incubated for an additional 2 days before being used for cytokine or RNA analysis.

**Cytokine measurement**

Upon the differentiation protocol, cells were cultured at 1 × 10^6/ml in 24-well plates overnight in the presence of PMA (20 ng/ml; Sigma-Aldrich) and ionomycin (1 μM; Sigma-Aldrich) to stimulate cytokine production. Supernatants were harvested, and IL-13 production was analyzed using the Quantikine M IL-13 ELISA kit according to manufacturer’s instructions (R&D Systems). Recombinant murine IL-13 (rmIL-13) was used as the standard. Briefly, supernatants and standard were diluted and applied to 96-well plates coated with polyclonal Ab specific for mouse IL-13. Plates were incubated at room temperature for 2 h and were developed with HRP-conjugated anti-mouse secondary anti-IL-13 Ab, followed by the addition of tetramethylbenzidine and hydrogen peroxide. OD450 was determined using an Emax microplate reader and SoftMax Pro 4.0 software ( Molecular Devices).

The production of IFN-γ, IL-4, and IL-5 was analyzed using the BD Mouse Th1/Th2 Cytokine Cytometric Bead Array (CBA; BD Pharmingen). A pooled mixture of rmIFN-γ, rmIL-5, and rmIL-4 diluted in assay diluent was used to create the standard curve. Individual Ab-conjugated capture beads for each cytokine of interest were pooled and incubated with diluted standard or samples and PE detection reagent, which consists of a mixture of PE-conjugated anti-murine IFN-γ, anti-murine IL-4, and anti-murine IL-5. The mixture of beads, standard or sample, and PE detection reagent was incubated for 2 h at room temperature. Individual cytokine concentrations were obtained by acquiring capture beads on the FACSCalibur and converting mean fluorescence intensities into concentration values by extrapolating from the standard curve using CBA software (BD Immunocyto-metry Systems).

**RT and real-time PCR analysis**

Differentiated CD4+ T cells were cultured at 5 × 10^6/ml on anti-CD3 (1 μg/ml)-coated, 6-well plates for 4 h at 37°C. RNA was isolated from cultured cells using the Tripure RNA isolation reagent protocol (Roche). RT of RNA was conducted for 60 min at 37°C using the Omniscript Reverse Transcriptase kit (Qiagen) and oligo(dT) primer. Gene expression was quantitated using TaqMan Universal PCR Master Mix (250 U of AmpliTaq Gold DNA polymerase, dUTP, dATP, dCTP, dGTP, 10× TaqMan buffer A, and 25 mM MgCl₂ solution) and 20× Assays-On-Demand Gene Expression Assay mixtures (one mix for each target gene, eukaryotic 18S (4319413E), murine Fas (Mm00433237_m1), murine FasL (Mm00438864_m1), or murine TRAIL (Mm00437174_m1) purchased from Applied Biosystems. Reactions consisting of 1× TaqMan Universal PCR Master Mix, 1× of the appropriate Assay-On-Demand gene mix (0.9 μM of each primer and 1× of all probe), and cDNA were prepared in 96-well optical reaction plates (Applied Biosystems). As an endogenous control, primers and probe for eukaryotic 18S were used. All PCRs for 18S and the target genes were performed in triplicate in the 7900 HT thermal cycler (Applied Biosystems) using the following default conditions: initial setup (50°C for 2 min, 95°C 10 min) and 40 cycles (95°C for 15 s, 60°C for 1 min).

Analysis of real-time PCR data was performed using the comparative CT method (ΔΔCT) with ABI PRISM SDS 2.1 software (Applied Biosystems). For each sample, the mean threshold cycle (CT) value was calculated for 18S and for the target gene of interest. The mean 18S CT value was subtracted from the mean target CT value to yield the ΔCT value. The ΔΔCT value was calculated by subtraction of ΔCT for the sample of interest by the ΔCT value for the calibrator (C57BL/6 control in the absence of secondary anti-CD3 stimulation). The ΔΔCT value was used to calculate the fold difference using the following arithmetic formula: fold difference = 2^ΔΔCT.

**AICD assays**

Cells were resuspended at 1 × 10^6/ml and plated onto fresh anti-CD3 (10 μg/ml)-coated, 24-well plates. Cells were incubated for 6 h at 37°C, harvested, washed, and fixed in 80% ethanol. Fixed cells were washed, resuspended in 400 μl of propidium iodide solution (50 μg/ml) containing 10 μg/ml RNase A (Roche), and incubated for 30 min at room temperature. The percentage of apoptotic cells was determined by analyzing the DNA content of propidium iodide-stained cells on the FACSCalibur using
CellQuest software. The percentage of apoptotic cells represented by hypodiploid (<2 N DNA content) cells was enumerated.

Statistical analysis

Statistical significance was assessed by paired two-tail Student’s t test and ANOVA using Microsoft Excel software. The ANOVA results presented for Fig. 1 are from one representative of several independent experiments.

Results

Morphine promotes Th2 development

Morphine has been shown to enhance the development of Th2 cells from unpurified splenocytes (11). To test whether the effects of morphine are exerted directly on T cells or indirectly through other cells, we analyzed the ability of morphine to enhance Th2 development in purified CD4+ T cell cultures. In all experiments, C57BL/6 CD4+ T cells were differentiated under neutral conditions using anti-CD3 and anti-CD28 (primary activation stimulus) and proliferation in IL-2 to allow for the development of Th1 and Th2 cells in the culture. In addition, cells were cultured in the presence or the absence of increasing concentrations of morphine. Morphine did not alter cell viability or recovery during the differentiation protocol (data not shown). After Th cell differentiation, cells were stimulated (secondary TCR activation) with PMA and ionomycin to drive cytokine production. As shown in Fig. 1A, increasing concentrations of morphine significantly enhanced IL-4 production after secondary TCR stimulation with a peak in IL-4 production between 30 and 100 nM. Morphine significantly enhanced IL-4 (F5,12 = 3.11; p < 0.0001) and IL-13 (F5,12 = 3.11; p < 0.0001; Fig. 1B) production compared with control cells, as determined by ANOVA. Although morphine was also found to enhance IL-5 and IFN-γ production (Fig. 1A), the increase in the concentrations of these cytokines was <2-fold. These results indicate that morphine enhances IL-4 and IL-13 production.

Morphine enhances Fas, FasL, and TRAIL mRNA expression in CD4+ T cells

Morphine was previously shown to enhance the mRNA expression of Fas in A1.1 T cell hybridoma and human PBL (23). We therefore tested the ability of morphine to promote Fas, FasL, and/or TRAIL mRNA expression in purified CD4+ T cells. The 100-nM concentration of morphine was chosen because this concentration produced significant changes in IL-4 and IL-13 production with no associated toxicity. After differentiation, cells were stimulated in the presence or the absence of plate-bound anti-CD3 (1 μg/ml) for 4 h. Fas, FasL, and TRAIL expression were analyzed by quantitative real-time PCR using the ABI comparative ΔΔCT method, and all values were normalized to untreated control cells. Anti-CD3 stimulation significantly enhanced the production of FasL (10-fold induction) and TRAIL (3-fold induction) expression by

![FIGURE 1](image1.png)

**FIGURE 1.** Morphine enhances IL-4 and IL-13 production by CD4+ T cells. Freshly purified CD4+ T cells were cultured under neutral conditions in the presence of increasing concentrations of morphine (10, 30, 100, 300, and 1000 nM) as described in Materials and Methods. On the sixth day of culture, cells (1 × 10^6 cells/ml) were activated overnight with PMA (20 ng/ml) and ionomycin (1 μM). A, IFN-γ, IL-4, and IL-5 were analyzed in supernatants by CBA; B, IL-13 was measured by ELISA. All samples were performed in triplicate. Values shown are the mean cytokine concentration ± SD from a representative of five independent experiments. The statistical significance of morphine-induced IL-4 and IL-13 production was assessed by ANOVA (F5,12 = 3.11; p < 0.0001 for both cytokines).

![FIGURE 2](image2.png)

**FIGURE 2.** Morphine enhances Fas, FasL, and TRAIL mRNA expression in CD4+ T cells. CD4+ T cells were cultured under neutral conditions in the presence or the absence of morphine (100 nM). On the sixth day of culture, cells were incubated with plate-bound anti-CD3 (1 μg/ml) at 1 × 10^5/ml for 4 h to induce gene expression. Cells that did not receive anti-CD3 treatment served as controls. Fas (A), FasL (B), or TRAIL (C) mRNA expression was analyzed by real-time PCR and ΔΔCT analysis as described in Materials and Methods. PCRs for target genes and the 18S rRNA endogenous control were performed in triplicate. Values shown represent the expression of the target gene normalized to the 18S endogenous control relative to control cells in the absence of anti-CD3 stimulation as a calibrator. Data presented are representative of three independent experiments.
control cells (Fig. 2, B and C). Conversely, the expression of Fas (Fig. 2A) was not further enhanced by secondary TCR stimulation of these cells. Morphine treatment did not significantly alter Fas, FasL, or TRAIL expression in the absence of a secondary TCR stimulus. However, in the presence of anti-CD3, morphine significantly enhanced the expression of Fas (2.5-fold induction), FasL (2.5-fold induction), and TRAIL (1.7-fold induction) compared with control cells treated with anti-CD3 without morphine. These results indicate that morphine enhances Fas, FasL, and TRAIL expression in CD4⁺ T cells activated through the TCR.

**Anti-FasL inhibits IL-4 and IL-13 production by CD4⁺ T cells**

Th1 cells are known to express high levels of FasL (21) and are susceptible to Fas-mediated death, whereas Th2 cells are relatively resistant (18). It was hypothesized that blocking the death of Th1 cells via Fas/FasL interaction could enhance IFN-γ production and inhibit Th2 development and IL-4 production. To test this possibility, CD4⁺ T cells were cultured in the presence or the absence of anti-FasL (10 μg/ml) and were restimulated with PMA and ionomycin to drive cytokine production. Treatment of CD4⁺ T cells during development with anti-FasL significantly inhibited the production of both IL-4 and IL-13 compared with control values (Fig. 3). Anti-FasL significantly reduced IL-4 (p < 0.05) and IL-13 (p < 0.05) production. The decrease in IL-4 production by anti-FasL was not associated with a concomitant enhancement of IFN-γ or IL-5 production (Fig. 3A).

**Anti-FasL inhibits morphine-induced IL-4 and IL-13 production**

Because morphine was shown to enhance FasL expression via a TCR-dependent mechanism, and FasL was shown to influence IL-4 and IL-13 production, we examined whether the enhancement of IL-4 and IL-13 production by morphine was Fas/FasL interaction dependent. CD4⁺ T cells were incubated with morphine (100 nM) in the presence or the absence of an inhibiting anti-FasL Ab (10 μg/ml). The morphine-enhanced IL-4 production was significantly inhibited by coculture with anti-FasL during differentiation (Fig. 4A). Similarly, the enhancement of IL-13 by morphine was inhibited by anti-FasL (Fig. 4B). These results indicate that the ability of morphine to enhance IL-4 and IL-13 production is dependent upon Fas-FasL interaction.

**Naltrexone inhibits morphine-induced gene expression and cytokine production**

To determine whether the effects of morphine were mediated through a classical opioid receptor, experiments were performed using the opioid receptor antagonist naltrexone in combination with morphine. Interestingly, naltrexone significantly inhibited the morphine-induced expression of Fas, FasL, and TRAIL, with complete reduction at a concentration as low as 10 nM (Fig. 5, A–C). At this 10-nM concentration, naltrexone also significantly inhibited the promotion of IL-4 (p < 0.005) and IL-13 (p < 0.05) production by morphine (Fig. 5, D and E). These results indicate that morphine enhances gene expression and cytokine production via a mechanism dependent upon Fas-FasL interaction.
Figure 5. Naltrexone inhibits morphine-induced gene expression and cytokine production. Fas (A), FasL (B), or TRAIL (C) mRNA expression was analyzed by real-time PCR and ΔΔCT analysis. CD4+ T cells were cultured under neutral conditions in the presence or the absence of morphine (100 nM) and/or increasing concentrations of naltrexone. On the sixth day of culture, cells were incubated with plate-bound anti-CD3 (1 μg/ml) at 1 × 10^6/ml for 4 h to induce gene expression. Cells that did not receive anti-CD3 treatment served as controls. Values shown represent expression of the target gene normalized to the 18S endogenous control relative to control cells. All data presented are representative of three independent experiments.

Discussion

Research over the past 20 years has increased our understanding of the factors that are critical to Th cell differentiation, including TCR signaling, the cytokine milieu, and activation of specific transcription factors. In addition, previous results from our group and others have revealed an important role for death effectors in governing the outcome of the Th cell differentiation process (15–18, 21). We demonstrated that morphine treatment can induce Fas expression in A1.1 T cell hybridoma and in primary human PBL (23). Morphine has previously been shown to enhance the production of IL-4 by total splenocytes after a short, 6-h incubation with anti-CD3 and anti-CD28 (11). Studies in vitro using unpurified splenocytes (24) and in vivo (25) have revealed that morphine depresses Ab formation. Additionally, morphine has been shown to promote ex vivo IL-12 cytokine production by macrophages when administered in vivo (26). However, the direct effects of morphine on CD4+ T cells cannot be readily deduced from experiments using mixed cell populations. Therefore, in this report we analyzed the ability of morphine to promote death effector expression and to influence Th2 differentiation in highly purified CD4+ T cells.

Morphine enhances AICD of CD4+ T cells

To determine whether increased gene expression correlated with increased protein expression and function, AICD assays were performed. Cells were differentiated under neutral conditions in the presence or the absence of morphine (100 nM). To promote AICD, primed cells were treated with plate-bound anti-CD3 (10 μg/ml) for 6 h. In addition, cells were incubated with anti-FasL (10 μg/ml) to inhibit AICD. The percentage of apoptotic cells was determined by DNA content analysis. As shown in Fig. 6, anti-CD3 stimulation promoted apoptosis of CD4+ T cells. However, the percentage of apoptotic cells in controls was significantly enhanced in the morphine-treated group (p < 0.005). The enhancement of apoptosis of CD4+ T cells by anti-CD3 was significantly inhibited by anti-FasL (p < 0.05). Similarly, anti-FasL significantly inhibited the promotion of AICD by morphine in the presence of anti-CD3 (p < 0.0005). These results indicate that morphine promotes FasL-dependent AICD of CD4+ T cells.
transcription in human T cells via binding of STAT-6 to putative IL-4-responsive elements within the promoter of the MOR gene (27). Therefore, by enhancing IL-4 production, morphine could also enhance the expression of the MOR on the surface of Th2 cells creating a positive feedback loop allowing for increased morphine binding and increased IL-4 production.

Another mechanism of action of morphine may be to differentially modulate helper cell survival. Morphine enhanced Fas, FasL, and TRAIL mRNA expression after secondary TCR activation in primary CD4+ T cells. Because Th1 cells are known to express high levels of FasL and low levels of TRAIL, whereas the converse is true for Th2 cells (18, 21), it is possible that morphine enhances FasL expression on Th1 cells and TRAIL expression on Th2 cells in these cultures. The enhancement of Fas, FasL, and TRAIL mRNA expression was completely inhibited by naltrexone, indicating that morphine is acting through a classical opioid receptor(s) to induce its effects on death effector expression. It is known that the expression of Fas requires protein kinase C (PKC) and expression of FasL requires both PKC and cytosolic Ca2+ (28). Because signaling through the MOR enhances cytosolic free Ca2+ levels in human embryonic kidney 293 cells (29, 30) and also induces PKC activation in the rat brain (31), it is possible that activation of PKC and cytosolic Ca2+ release by morphine may lead to enhanced Fas and FasL expression during TCR activation.

Interestingly, resting splenic T cells have also been shown to express δ opioid receptor (DOR) mRNA transcripts whose expression can be enhanced or inhibited depending upon the use of anti-CD3 or PMA and ionomycin, respectively, to stimulate T cells (32, 33). Signaling via this DOR by both β-endorphin and DOR agonists has been shown to enhance intracellular Ca2+ mobilization by Con A-stimulated splenic T cells (34, 35). It is possible that morphine may be enhancing calcium flux, and therefore death effector gene expression, in CD4+ T cells via binding to the DOR. Additional experiments using selective opioid receptor antagonists or opioid receptor knockout mice are necessary to determine the specific receptor used by morphine in this experimental setting.

Because morphine was shown to significantly enhance IL-4 and IL-13 production and FasL expression, the role of FasL in morphine-induced cytokine production was examined. Blockade of FasL was shown to significantly inhibit morphine-induced IL-4 and IL-13 production, indicating that morphine enhances Th2 development and cytokine production by a FasL-dependent mechanism. Interestingly, naltrexone, which was found to inhibit morphine-induced FasL expression, also inhibited morphine-induced IL-4 and IL-13 production. These results indicate that morphine enhances FasL expression via binding to a classical opioid receptor, and enhanced gene expression ultimately alters cytokine production. These FasL data can be interpreted in two ways. One interpretation is that FasL may promote morphine-induced Th2 development directly, and therefore blockade of FasL inhibits Th2 development. Another interpretation is that FasL expression may be involved in the suppression of Th1 development and therefore indirectly promotes Th2 development. Because Th2 cells are known to express low levels of FasL (18, 21), it is unlikely that FasL signaling in Th2 cells would account for the increased IL-4 and IL-13 production observed. Additionally, Th1 cells are known to express high levels of Fas and FasL and are susceptible to Fas-mediated apoptosis. Therefore, it is possible that the killing of nascent Th1 cells via Fas/FasL interaction could account for the outgrowth of IL-4-producing Th2 cells. Indeed, the presence of anti-FasL throughout the Th cell differentiation process was found to inhibit the development of naive Th cells into Th2 cells, as evidenced by a decrease in IL-4 and IL-13 production. This finding is supported by previous work in our laboratory indicating that blockade of FasL using anti-FasL Ab inhibits IL-4 production by CD4+ T cells cultured under neutral conditions (21). However, this model would predict a concomitant change in IFN-γ production, but we did not observe a dramatic change in IFN-γ. Thus, characterization of the FasL-dependent mechanism by which morphine enhances IL-4 and IL-13 production requires additional exploration.

To determine the functional significance of the increased Fas and FasL mRNA expression induced by morphine and whether Fas-mediated apoptosis is important to morphine’s ability to promote Th2 development, AICD assays were performed. The results revealed that morphine could significantly promote AICD of CD4+ T cells in response to a secondary stimulus through the TCR. Secondary TCR activation is known to enhance FasL expression on the cell surface of Th1 cells and promote the susceptibility of Th1 cells to AICD (15). We found that AICD induced by morphine was completely inhibited by anti-FasL Ab, suggesting that the majority of T cells killed in the cultures are Th1 cells, because Th2 cells are known to be resistant to Fas-mediated apoptosis (17, 18). The findings presented indicate that in the absence of exogenous cytokines, a number of players are involved in the development of naive CD4+ T cells, including death effectors (Fas, FasL, and TRAIL) and opioids. Interestingly, morphine has also been shown to induce apoptosis of macrophages both in vivo and in vitro (36, 37) and of human peripheral blood mononuclear leukocytes in vitro (38). Taken together with our results, these findings reveal that morphine has the ability to modulate multiple immune responses by regulating the survival and function of both macrophages and T cells.

The ability of opioids, such as morphine, to influence naive CD4+ T cells toward the Th2 cell fate provides additional support for an interaction between the nervous and immune systems and aids in understanding the complex mechanisms that govern the fate of Th cells. The observation that morphine promotes Th2 cytokine production can help to explain why chronic opioid use often leads to immune suppression to infections and enhanced asthma (39–

![Figure 6](image_url)


