Morphine Disrupts Interleukin-23 (IL-23)/IL-17-Mediated Pulmonary Mucosal Host Defense against *Streptococcus pneumoniae* Infection[▽]

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Streptococcus pneumoniae is a pathogen that causes serious respiratory disease and meningitis in the immunocompromised drug abuse population. However, the precise mechanisms by which drug abuse compromises the host immune defense to pulmonary *S. pneumoniae* infection is not fully understood. Using a well-established murine model of opiate abuse and *S. pneumoniae* lung infection, we explored the influence of morphine treatment on the interleukin-23 (IL-23)/IL-17 axis and related innate immunity. Impairment of early IL-23/IL-17 production caused by morphine treatment was associated with delayed neutrophil migration and decreased pneumococcal clearance. Furthermore, morphine treatment impaired MyD88-dependent IL-23 production in alveolar macrophages and dendritic cells in response to *in vitro S. pneumoniae* cell infection. Moreover, morphine treatment significantly inhibited the *S. pneumoniae*-induced phosphorylation of interferon response factor 3 (IRF3), ATF2, and NF- κ Bp65. T-cell receptor δ (TCR δ)-deficient mice showed a decrease in IL-17 production and a severely weakened capacity to clear lung *S. pneumoniae* infection. Finally, morphine treatment resulted in diminished secretion of antimicrobial proteins S100A9 and S100A8/A9 during early stages of *S. pneumoniae* infection. In conclusion, morphine treatment causes a dysfunction in IL-23-producing dendritic cells and macrophages and IL-17-producing $\gamma\delta$ T lymphocytes in response to *S. pneumoniae* lung infection. This leads to diminished release of antimicrobial S100A8/A9 proteins, compromised neutrophil recruitment, and more-severe infection.

Immunocompromised individuals are at high risk for *Streptococcus pneumoniae* pulmonary infection (30). Previous studies have shown that opiate abuse causes immunosuppression by disrupting both innate and adaptive components of the immune system (26, 33). Opiate abuse is a critical risk factor for increasing susceptibility and severity of bacterial infection, including *S. pneumoniae* (31, 32). However, additional work is needed to understand the precise mechanisms by which opiate abuse increases the susceptibility to *S. pneumoniae* lung infection.

Interleukin-23 (IL-23) has been recently identified as a cytokine closely related to IL-12 (5). The balance between IL-23 and IL-12 controls the outcome of inflammatory responses (16). IL-23 is secreted by activated macrophages and dendritic cells (DCs) and induces memory T-cell proliferation and is the critical factor required for T-cell IL-17 expression in response to bacterial challenge (3). IL-23 release leads to the production of IL-17. Furthermore, IL-17 promotes neutrophilic inflammation by upregulating CXC chemokines and hematopoietic growth factors (13). Several recent studies report the important role of IL-23 and IL-17 in the induction of neutrophil-

mediated protective immune response against extracellular bacterial or fungal pathogens, such as *Klebsiella pneumoniae* (14), *Pseudomonas aeruginosa* (12), *Porphyromonas gingivalis* (34), *Citrobacter rodentium* (20), *Bacteroides fragilis* (9), and *Escherichia coli* (27).

Generally, the IL-23/IL-17 axis plays an important role in the host defense against bacterial infections (11). Previous studies have demonstrated that IL-17 is critical for the recruitment of phagocytes that leads to the clearance of *S. pneumoniae* colonization from the mucosal surface of the nasopharynx (18, 35). However, whether the IL-23/IL-17 axis contributes to modulating the innate immunity in response to *S. pneumoniae* lung infection has not been addressed. Using a well-established opiate abuse and *S. pneumoniae* lung infection mouse model (31, 32), we demonstrate that *S. pneumoniae* induces IL-23 and IL-17 expression in the lungs as early as 2 h following infection. Morphine treatment causes a decrease in both IL-23 and IL-17 synthesis during the early stages of infection, leading to delayed neutrophil recruitment. This results in an increased bacterial burden within the lungs and the initiation of systemic disease.

MATERIALS AND METHODS

Experimental animals. Pathogen-free B6129PF1 and B6.129P2 T-cell receptor δ (TCR $\delta^{-/-}$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific-pathogen-free (SPF) facility under barrier conditions. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the University of Minnesota.

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Pneumococcal pneumonia infection and morphine treatment protocol. A murine opiate abuse and pneumococcal pneumonia model has been used extensively by our laboratory as previously described (31, 32). In brief, the virulence of this organism was maintained by subculturing bacteria obtained from the spleens of bacteremic mice and storing them at -80°C until use. For culture growth of the bacteria, *S. pneumoniae* serotype 3 (ATCC 6303; Rockville, MD) were streaked onto a blood agar plate (Becton, Dickinson and Co.) and grown overnight at 37°C . Typical colonies were picked and inoculated into brain heart infusion (BHI) broth. The culture was incubated for 6 h at 37°C to produce log-phase organisms. The bacteria were pelleted by centrifugation and washed twice in endotoxin-free phosphate-buffered saline (PBS; Invitrogen). The concentration of bacteria was determined spectrophotometrically (OD_{590}) and confirmed by plating serially diluted bacteria onto blood agar plates. Mice were lightly anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ) and inoculated with approximately 10^7 CFU of *S. pneumoniae* in $50\ \mu\text{l}$ of PBS applied to the tip of the nose and involuntarily inhaled. The animals were held in a vertical position for 1 to 2 min, ensuring migration of the inoculum to the alveoli.

Mice were subcutaneously implanted with either a 75-mg morphine slow-release pellet (National Institute on Drug Abuse [NIDA], Rockville, MD) or placebo pellet (controls) 24 h before *S. pneumoniae* inoculation. The plasma levels achieved following morphine pellet implantation is in the 200- to 350-ng/ml range at steady state for 7 days. Plasma morphine concentrations in patients that are administered morphine for mild to severe pain are in the 20- to 2,000-ng/ml range, while morphine levels in heroin drug abusers are in the 200- to 2,000-ng/ml range. The doses of morphine used in our studies are well within the range observed in the plasma of patients that are administered morphine for moderate to severe pain and in heroin abusers (8, 10, 21, 25, 28).

To assess the effect of IL-17 administration on bacterial load, 15 ng of recombinant murine IL-17 (R&D Systems) was delivered via the intranasal route, in a volume of $50\ \mu\text{l}$ of sterile PBS, following light isoflurane anesthesia 2 h after infection. Mice were sacrificed 24 h postinfection to collect the lung tissue and bronchoalveolar lavage (BAL) fluid samples as well as blood samples in order to test bacterial burden and dissemination.

BAL and neutrophil enumeration in BAL fluid and lung tissue. The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott). BAL fluid samples were obtained by instilling and collecting 1-ml volumes of cold PBS through the incised trachea. A total of 0.9 ml of lavage fluid was retrieved per mouse. Total cell numbers in the BAL fluid samples were counted from each sample with a hemocytometer (Hausser Scientific, Horsham, PA). The BAL fluid neutrophil count was determined on cytopsin preparations stained with a Diff-Quick staining kit (IMEB). The neutrophil population in the lung tissue samples was evaluated by the quantification of myeloperoxidase (MPO) activity (31, 32).

Pathogenesis studies. Mice were sacrificed by CO_2 asphyxiation at 4, 24, and 48 h postinfection. Blood samples were collected through the heart, and $10\ \mu\text{l}$ of blood was plated onto a blood agar plate and incubated at 37°C overnight to determine the presence of bacteria. BAL fluids were obtained as described above. Lungs were aseptically removed, cleared of blood with cold saline, and homogenized in 2 ml of cold PBS. Serial dilutions of the lung homogenates and BAL fluid samples were plated onto blood agar plates. Plates were incubated at 37°C overnight, and *S. pneumoniae* colonies were counted.

Cells, *in vitro* morphine treatment, and cell infection. The following cells were used for the *in vitro* experiments.

Resident alveolar macrophages (AMs) from mice were obtained via *ex vivo* lung lavage and resuspended in RPMI 1640 to a final concentration of 1×10^6 cells/ml. Cells were allowed to adhere to plates for 1 h (37°C , 5% CO_2), followed by two washes with warm RPMI 1640, resulting in $>99\%$ of adherent cells identified as AMs by use of a Diff-Quick stain.

Bone marrow-derived DCs (BMDCs) were generated by following a previously described method (19).

AMs and BMDCs (1×10^6) were treated for 24 h in media containing vehicle (PBS control) or various concentrations of morphine (10 nM to $1\ \mu\text{M}$; NIDA, Rockville, MD). Then, cells were infected with *S. pneumoniae* (10^7 CFU; multiplicity of infection [MOI], 10:1), for various periods of time, depending on the experiment.

To determine whether naltrexone (Sigma) antagonizes morphine's actions, naltrexone was used at a concentration of $10\ \mu\text{M}$ to pretreat cells for 1 h before morphine treatment.

To inhibit the MyD88-dependent signaling, cells were pretreated with MyD88 homodimerization inhibitory peptide or control peptide (Imgenex) at 24 h before *in vitro* cell infection with *S. pneumoniae*. The inhibitory peptide contains a

sequence from the MyD88 TIR homodimerization domain, which specifically inhibits the MyD88-dependent pathway (17).

ELISA. The levels of IL-23 and IL-17 in the BAL fluids and lung tissues of *S. pneumoniae*-infected mice and cell culture supernatant of *in vitro* infected cells were quantified using cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Quantification of S100A9 and S100A8/A9 was done using ELISA as previously described by Vandal et al. (29).

Western blotting and native PAGE for analysis of IRF3 dimerization. For SDS-PAGE and subsequent Western blotting, 1×10^6 adherent BMDCs were lysed using M-PER mammalian protein extraction reagent (Pierce) supplemented with a set of protease inhibitors (Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich). Nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's instruction. Proteins were separated on an SDS gel and transferred onto a nitrocellulose membrane (Amersham Biosciences). Membranes were washed in Tris-buffered saline-Tween 20 (TBST), blocked for 1 h in StartingBlock blocking buffer, and then incubated overnight at 4°C in the primary antibodies, p-NF- κB p65, p-activating transcription factor 2 (ATF2) (Cell Signaling), and interferon response factor 3 (IRF3; Santa Cruz). The blots were then incubated for 1 h at room temperature with anti-rabbit secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Western blot analysis was conducted according to standard procedures, using SuperSignal chemiluminescence detection substrate (Pierce). Signals were detected by an UltraQuant image acquisition and analysis system. To verify the quality of loading, the blots were reprobbed with anti- β -actin (Santa Cruz) or anti-TATA binding protein (Abcam).

Native PAGE for analysis of the IRF3 dimerization experiment was performed as described by others (15). Briefly, $50\ \mu\text{g}$ of nondenatured whole-cell protein extracts were loaded onto a preelectrophoresed 4.5% polyacrylamide gel and separated at 350 V at 4°C . Transfer and Western blotting were done as described above.

Real-time RT-PCR. Total RNA from lung homogenates and BAL fluid cells was isolated using the RNeasy mini kit (Qiagen). Ten nanograms of total RNA was subjected to two-step reverse transcription-PCR (RT-PCR). Real-time SYBR green PCR analysis was performed for IL-23p19 (sense, 5'-CCAGCGG GACATATGAATCT-3'; antisense, 5'-AGGCTCCCTTTGAAGATGT-3') and IL-17 (sense, 5'-TCTCTGATGCTGTTGCTGCT-3'; antisense, 5'-CGTGG AACGGTTGAGGTAGT-3') mRNA and 18S rRNA. Relative quantification was performed using the ABI Prism 7500 sequence detection system (Applied Biosystems). IL-23p19 and IL-17 transcript levels were normalized to 18S rRNA transcript levels from the same preparations of cDNA.

Statistical analysis. Data were collected from three independent experiments and expressed as the mean $\pm$ standard error of the mean (SEM). Where appropriate, mean values were compared using a paired Student's *t* test or two-way analysis of variance (ANOVA). A *P* value of <0.05 was considered statistically significant.

RESULTS

IL-23 and IL-17 expression during *S. pneumoniae* lung infection. To determine the role of IL-23/IL-17 in host defense against respiratory *S. pneumoniae* infection, IL-23/IL-17 protein and mRNA levels following *S. pneumoniae* infection in the lung tissue and BAL fluid samples and cells were quantified. As shown in Fig. 1A, IL-23 was rapidly released as early as 2 h following *S. pneumoniae* infection in both the lung tissues and BAL fluids, was significantly elevated to peak levels at 4 h, and declined by 24 h postinfection. In the lung tissues, IL-17 was detected within 2 h after *S. pneumoniae* infection and peaked at 4 h postinfection. This was followed by a general decrease, but the levels remained elevated until 24 h postinfection. In the BAL fluids, IL-17 was not detectable during the early stages of infection (2, 4, and 6 h postinfection) and peaked at 24 h postinfection (Fig. 1B). Furthermore, mRNA levels of IL-23 and IL-17 in the lung tissues was markedly upregulated by *S. pneumoniae* infection as early as 2 h and remained elevated at

FIG. 1. Protein production and mRNA expression of IL-23 and IL-17 in the lungs, BAL fluids, and cells after *S. pneumoniae* infection. Mice were intranasally infected (10^7 CFU per mouse) with *S. pneumoniae* (serotype 3). At various time points postinfection, lung tissue and BAL fluid samples were collected. The concentration of IL-23 (A) and IL-17 (B) in lung homogenates and BAL fluids was measured by ELISA. The mRNA expression of IL-23 (C) and IL-17 (D) in the lung tissues and BAL cells was determined by real-time RT-PCR. Data represent the mean $\pm$ SEM of the results for three independent experiments ($n \geq 6$ mice per group).

24 h. Similar to IL-17 protein level, IL-17 mRNA level in the BAL cells was observed only 24 h after *S. pneumoniae* infection (Fig. 1C and D). Our previous studies found that significant leukocyte recruitment to the lung occurred only after 6 h following *S. pneumoniae* infection (31, 32), suggesting that both IL-23 and IL-17 were produced by lung resident cells and not by recruited cells in the early stages of *S. pneumoniae* infection. The rapid release of IL-17 and IL-23 following pneumococcal infection may be due to initial release of preformed cytokines. However, we also observed a rapid increase in IL-23 and IL-17 mRNA levels, so the role of transcriptional activation in the sustained synthesis of the cytokines cannot be ruled out.

Morphine treatment disrupts pulmonary IL-23 and IL-17 expression in the early stages of *S. pneumoniae* lung infection.

To address whether morphine disrupts the IL-23/IL-17 axis, leading to compromised neutrophil recruitment and more-severe infection, morphine- and placebo-treated mice were infected with *S. pneumoniae*, and IL-23/IL-17, neutrophil migration, and bacterial burden were measured in the lungs at various time points. As shown in Fig. 2A to D, morphine treatment caused a decrease in both IL-23 and IL-17 synthesis during the early stages of infection. This decrease in IL-23 and IL-17 production was associated with delayed and decreased neutrophil recruitment into BAL fluid and lung tissues following *S. pneumoniae* infection, increased bacterial burden within the lungs, and the initiation of systemic disease. These results indicate that a rapid IL-23/IL-17-mediated neutrophil re-

sponse participates in the initial control of *S. pneumoniae* infection. These studies also suggest that morphine treatment causes suppression of the IL-23/IL-17 axis, leading to a decrease in neutrophil recruitment and pneumococcal clearance.

Effect of rIL-17 on clearance of *S. pneumoniae* infection. To determine the role of IL-17 in host defense against *S. pneumoniae*, we performed a rescue experiment using recombinant murine IL-17 (rIL-17; 15 ng/mouse) administered intranasally 2 h after infection, the time at which IL-17 becomes detectable in the lung tissues in this infection model. Mice were sacrificed at 24 h postinfection. Morphine-treated mice showed significantly higher burdens of *S. pneumoniae* in the lung tissues as well as greater dissemination to the blood 24 h postinfection in comparison with placebo-treated control mice. Administration of IL-17 significantly improved lung antibacterial host defense and reduced bacterial dissemination to the blood (Fig. 3). IL-17 treatment significantly reduced the high bacterial burden observed in morphine-treated mice, further suggesting a critical role for IL-17 in host defenses in the opiate abuse and *S. pneumoniae* lung infection model.

Morphine treatment impairs AM and DC IL-23 production in response to *in vitro* cell infection. To directly determine which cell populations among lung resident cells produce IL-23 and IL-17 in response to *S. pneumoniae* infection and, more importantly, to determine whether morphine suppresses this response independent of the presence of other cell types, primary mouse AMs and BMDCs were treated with morphine or

FIG. 2. Levels of IL-23 (A) and IL-17 (B), recruitment of neutrophils (C), and bacterial burden (D) in the lung tissues and BAL fluids in morphine-treated and placebo-treated control mice. Data are expressed as the mean $\pm$ SEM of the results for three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with the placebo-treated control group; $n \geq 6$).

vehicle for 24 h and then infected *in vitro* with *S. pneumoniae* (MOI, 10:1). As shown in Fig. 4A, *S. pneumoniae* infection stimulated IL-23 production in both AMs and DCs. However, the levels of IL-23 produced by DCs were markedly higher than those produced by macrophages. Our results suggest that both AMs and DCs contribute to IL-23 production in response to *S. pneumoniae* infection. However, DCs may be the primary IL-23-producing cell population responding to *S. pneumoniae* infection. Furthermore, morphine treatment in the higher dose range (1 μ M) inhibited this response in BMDCs, while morphine doses in the 10 or 100 nM range had no effect (Fig. 4A).

FIG. 3. Administration of rIL-17 improves bacterial clearance in morphine-treated mice. Mice were infected with 10^7 CFU of *S. pneumoniae* followed by intranasal administration of 15 ng rIL-17 (or vehicle) 2 h later. Animals were then sacrificed 24 h postinfection. Error bars represent mean $\pm$ SEM. **, $P < 0.01$.

Micromolar concentrations of morphine are typical drug abuse doses and concentrations achieved in patients that are prescribed morphine for moderate to severe pain. Plasma levels that are in the nanomolar range are generally observed in patients that receive morphine for mild and acute pain (8, 10, 21, 25, 28). The morphine action on *S. pneumoniae*-induced IL-23 was abolished by naltrexone, a prototypical opioid receptor antagonist, indicating that classical opioid receptors participate in the inhibition of IL-23 production caused by morphine following *S. pneumoniae* infection (Fig. 4B).

Morphine inhibits *S. pneumoniae*-induced IL-23 production through the MyD88-dependent IRF3, ATF2, and NF- κ B signaling. To determine if TLR-MyD88-dependent signaling is essential for IL-23 production following infection, and whether morphine treatment compromises this signaling in IL-23-producing cells, BMDCs were treated with morphine (1 μ M) or vehicle (control), plus MyD88 inhibitory peptide or control peptide, in a dose-dependent manner (10 nM to 100 μ M) for 24 h and then *in vitro* infected with *S. pneumoniae*. After 6 h of cell infection, cell culture supernatant was collected and used to detect IL-23 release by ELISA. As shown in Fig. 4C, treatment of BMDCs with various doses of MyD88 inhibitor resulted in decreased IL-23 production in a dose-dependent manner, indicating that the IL-23 production induced by *S. pneumoniae* infection is a downstream event that depends on MyD88 activation. Morphine treatment caused a significant decrease in *S. pneumoniae*-induced IL-23 production in BMDCs when pretreated with MyD88 control peptide. However, no difference in IL-23 production was observed between morphine- and vehicle-treated BMDCs when pretreated with 100 μ M MyD88 inhibitory peptide (Fig. 4C). This finding implies

FIG. 4. Effect of morphine treatment on pneumococcus-induced MyD88-dependent IL-23 production and phosphorylation of IRF3, ATF2, and NF- κ Bp65. (A) Mouse BMDCs and AMs (1×10^6) were treated with morphine (1 μ M) or vehicle for 24 h and then infected with *S. pneumoniae* for 6 h, and IL-23 concentration was measured in the supernatant by ELISA. **, $P < 0.01$, compared with the vehicle controls. Results are representative of three independent experiments. (B) BMDCs were pretreated with naltrexone (10 μ M) or vehicle for 1 h before morphine treatment (10 nM to 1 μ M) and then infected with *S. pneumoniae* (MOI, 10:1) for 6 h. IL-23 concentration was measured in the supernatant by ELISA. **, $P < 0.01$, compared with the vehicle controls. Results are representative of three independent experiments. (C) BMDCs were pretreated with MyD88 inhibitory or control peptide as described in Materials and Methods and then treated with either morphine (1 μ M) or vehicle for 24 h and infected with *S. pneumoniae* for 6 h. The data are presented as mean concentration $\pm$ SEM. **, $P < 0.05$ compared with the vehicle control group; $n \geq 6$. (D) Levels of p-IRF3 were compared by naive PAGE and immunoblotting. Phosphorylation of ATF2 and NF- κ Bp65 was compared by Western blot analysis. To verify equality of loading, blots were reprobed with anti- β -actin or anti-TATA binding protein (TBP). Shown are representative results from one of three independent experiments.

that morphine treatment impairs MyD88-dependent IL-23 production following *S. pneumoniae* infection.

To determine if unique signaling pathways are activated by *S. pneumoniae* infection, thereby accounting for the IL-23 expression, downstream signaling elements from MyD88 were evaluated. To investigate if inhibition of *S. pneumoniae*-induced IRF3, activating transcription factor 2 (ATF2), and NF- κ B activity is a critical mechanism by which morphine inhibits IL-23 production following infection, we examined whether morphine treatment had any effect on the phosphorylation status of IRF3, ATF2, and NF- κ B in *S. pneumoniae*-infected BMDCs. The phosphorylation of IRF3 at its C terminus, particularly on Ser-386 and Ser-396, is required for its dimerization and subsequent translocation to the nucleus (2). As shown in Fig. 4C, treatment of BMDCs with morphine reduced IRF3 phosphorylation and led to decreased induction of IRF3 dimerization in response to *S. pneumoniae* infection. Additionally, ATF2 and NF- κ Bp65 phosphorylation was induced, respectively, following *S. pneumoniae* cell infection. Morphine treatment significantly inhibited *S. pneumoniae*-in-

duced ATF2 and NF- κ Bp65 phosphorylation (Fig. 4C). These results suggest a significant role for MyD88-IRF3, ATF2, and NF- κ B signaling pathways in mediating morphine's inhibition of IL-23 production.

$\gamma\delta$ T cells contribute to IL-17 expression in response to *S. pneumoniae* lung infection. To determine the role of IL-17-producing $\gamma\delta$ T lymphocytes in *S. pneumoniae* infection and whether morphine treatment modulates IL-17-producing $\gamma\delta$ T cells, we compared the production of IL-23/IL-17 in the lungs following *S. pneumoniae* infection in TCR $\delta^{-/-}$ (B6.129P2) mice and strain-, age-, and gender-matched wild-type (WT; B6.129PF1) mice. Interestingly, *S. pneumoniae* infection induced IL-23 production in the BAL fluids and lung tissues of both WT and TCR $\delta^{-/-}$ mice. Morphine treatment diminished IL-23 production in the early stages of *S. pneumoniae* infection (Fig. 5A). However, significant levels of IL-17 production, induced by *S. pneumoniae* infection, were detected only in the lungs derived from WT mice and not in the lungs of TCR $\delta^{-/-}$ mice. Morphine significantly impaired IL-17 production in *S. pneumoniae*-infected WT mice. These results suggest that $\gamma\delta$ T

FIG. 5. Effect of morphine treatment on IL-23/IL-17 production and bacterial clearance in TCR $\delta^{-/-}$ and WT mice. IL-23 and IL-17 concentrations are shown at 4 h after intranasal infection with *S. pneumoniae*. Bacterial burden is shown at 24 h following *S. pneumoniae* infection. Each value represents the mean $\pm$ SEM. Mean values from three independent experiments are shown. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$, compared with the placebo controls.

cells are an essential IL-17-producing cell population in response to *S. pneumoniae* infection and that morphine treatment causes a dysfunction in pulmonary IL-17-producing $\gamma\delta$ T cells, leading to a defect in innate immunity (Fig. 5B). Consistent with IL-17 production, $\gamma\delta$ T-cell-deficient mice have a severely weakened capacity to clear *S. pneumoniae* lung infection. The number of live bacteria in the lungs was significantly higher ($P < 0.01$) in TCR $\delta^{-/-}$ mice than that in WT mice 24 h postinfection. Morphine treatment in the WT mice resulted in a greater bacterial burden in the BAL fluid and lungs than it did in placebo-treated WT mice. Interestingly, no difference in bacterial burden was observed between morphine and placebo treatment of TCR $\delta^{-/-}$ mice (Fig. 5C). These results clearly reveal that the IL-17-producing $\gamma\delta$ T cells play a critical role in the host resistance to pneumococcal infection and may be involved in morphine's actions.

Morphine inhibits the secretion of antimicrobial proteins S100A9 and S100A8/A9. To determine whether morphine treatment compromises antimicrobial proteins, S100A8/A9 proteins were quantified in the lung tissue and BAL fluid samples derived from mice infected with *S. pneumoniae*. As shown in Fig. 6, morphine treatment markedly decreased S100A9 and S100A8/A9 production in the early stages of *S. pneumoniae* infection. Secretion of S100 proteins preceded infiltration of neutrophils into the lung and alveolar space (23). These results suggest that morphine treatment may diminish the host release of antimicrobial proteins S100A8/A9, leading to decreased neutrophil recruitment and more-severe infection.

DISCUSSION

The current study demonstrates, for the first time to our knowledge, that morphine disrupts the IL-23/IL-17 axis, which diminishes the host release of antimicrobial peptides S100A8/A9 and, consequently, decreases neutrophil recruitment in response to *S. pneumoniae* infection, thus leading to more-severe infection.

Resident AMs and DCs, along with recruited macrophages and neutrophils, play a key role in the clearance of invading *S. pneumoniae* (22). While AMs are the primary resident cells in the BAL fluid, both DCs and AMs contribute to first-line cellular defense and cytokine secretion at early stages of infection in the lung tissues. Since AMs are key resident immune cells in both lung tissues and BAL fluid, and the main source of early proinflammatory cytokines released in the lungs, we expected AMs to be the major early source of IL-23 production following *S. pneumoniae* infection. However, our data show that following *S. pneumoniae* infection, lung tissue levels of IL-23 were significantly greater than that observed in BAL fluids, indicating that cells other than AMs may be the major early source of IL-23 following *S. pneumoniae* infection. Consistent with this observation, *in vitro* infection of DCs and AMs with *S. pneumoniae* reveals that although both DCs and AMs produce IL-23 in response to infection, DCs' production of IL-23 in response to *S. pneumoniae* infection was significantly greater than AMs'. These data imply that DCs are the primary early source of IL-23 following *S. pneumoniae* infection.

IL-17 and IL-17F are produced by $\alpha\beta$ T cells called Th17 cells, as well as by $\gamma\delta$ T cells (24). IL-17 is a cytokine that induces neutrophil-mediated inflammation, but its role in pro-

FIG. 6. Effect of morphine treatment on the secretion of S100A9 and S100A8/A9 during the early stages of *S. pneumoniae* infection. At various times postinfection, the lungs and BAL fluid samples were collected. The concentrations of S100A9 and S100A8/A9 were measured by ELISA in the lung homogenates and BAL fluids. Data represent the mean $\pm$ SEM of the results for ≥ 6 mice per group. *, $P < 0.05$; **, $P < 0.01$, compared with the placebo treatment group.

protective immunity against *S. pneumoniae* infection remains unclear. Our studies show that IL-17 expression in the *S. pneumoniae*-infected lung was detected within a few hours after infection and is IL-23 dependent. Interestingly, $\gamma\delta$ T-cell-deficient mice have a severely impaired capacity to clear lung *S. pneumoniae* infection. Moreover, $\gamma\delta$ T-cell-deficient mice showed a significant reduction in cellular infiltration into the airways and expression of IL-17 in the lung following *S. pneumoniae* infection (6). Our observations suggest that $\gamma\delta$ T cells may be a predominant source of IL-17 in the lungs following *S. pneumoniae* infection. Furthermore, no difference in bacterial outgrowth and dissemination was observed between morphine- and placebo-treated groups in TCR δ -deficient mice. Therefore, we conclude that IL-17-producing $\gamma\delta$ T cells may be a critical cell population participating in morphine treatment-caused increased susceptibility to *S. pneumoniae* infection. Our study, therefore, expands on the findings by Lu et al. (18) and Zhang et al. (35) that report that during the natural course of pneumococcal colonization, Th17 cells are critically required

for the recruitment of phagocytes that leads to the clearance of colonization in both naive and immune hosts. Our findings further demonstrate that IL-17-producing $\gamma\delta$ T cells are critical for the innate immune response against *S. pneumoniae* lung infection.

MyD88 significantly contributes to host defense mechanisms that control colonization of the respiratory tract and prevent invasive infection and proliferation of *S. pneumoniae* in the blood. MyD88 signaling is required for local cytokine production, leukocyte infiltration, and tissue destruction (1). Our results indicate that IL-23 production in response to *S. pneumoniae* infection requires MyD88 and that morphine treatment impairs MyD88-dependent IL-23 production. Furthermore, potential binding sites for IRF-3, Sma- and Mad-related protein 3 (SMAD-3), ATF2, IRF7, and NF- κ B have been identified in the murine IL-23p19 promoter (2). In both macrophages and DCs, IL-23p19 expression is dependent on the binding of c-Rel and RelA NF- κ B to the proximal IL-23p19 promoter (7). More recent promoter analyses reveal that besides a site for NF- κ B, regulatory elements for IRF3 and ATF2 at the p19 promoter are essential for promoter activity (2). In this study, we show that morphine treatment decreases *S. pneumoniae*-induced phosphorylation of IRF3, ATF2, and NF- κ Bp65, suggesting that inhibition of IRF3, ATF2, and NF- κ B signaling pathways is a critical mechanism by which morphine inhibits IL-23 production in *S. pneumoniae*-infected DCs.

There is accumulating evidence that IL-17 regulates the production of antimicrobial proteins in endothelial and epithelial cells (4). Antimicrobial proteins S100A8 and S100A9 are expressed by neutrophils, monocytes, and activated endothelial and epithelial cells. They regulate macrophage and neutrophil transepithelial migration from the lung tissue to the alveolar space as a host response to *S. pneumoniae* infection (23). The data presented herein show that morphine treatment decreased S100A9 and S100A8/A9 production during the early stages of *S. pneumoniae* infection. Decreased secretion of S100A8/A9 proteins was associated with reduced infiltration of neutrophils into the lung and alveolar space. Taken together, our study suggests that morphine treatment diminishes the host release of antimicrobial proteins S100A8/A9, which may lead to decreased neutrophil recruitment in response to *S. pneumoniae* infection.

Findings from this study indicate that IL-23 is produced by DCs and macrophages within a few hours after exposure to *S. pneumoniae*. This, in turn, triggers rapid IL-17 responses from lung resident $\gamma\delta$ T cells. IL-17 may promote the production of antimicrobial proteins S100A8/A9. In our previous studies, we have shown that *S. pneumoniae* infection results in the early release of MIP-2, KC, IL-1, and tumor necrosis factor alpha (TNF- α) (31, 32), resulting in rapid recruitment of neutrophils to the site of infection. Our current findings indicate that activation of the IL-23/IL-17 axis may be important in driving an early immune response against *S. pneumoniae*. Morphine treatment disrupts the IL-23/IL-17 axis, leading to diminished host release of antimicrobial proteins S100A8/A9. This results in decreased neutrophil recruitment and more-severe infection. The data generated from this study add to a new understanding of mechanisms by which morphine interferes with pulmonary innate immune defenses against *S. pneumoniae* in-

fection. This study also helps us to identify potential innate immunity-based therapies and prevention strategies against *S. pneumoniae* infection in the opioid abuse population.

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REFERENCES

- Albiger, B., A. Sandgren, H. Katsuragi, U. Meyer-Hoffert, K. Beiter, F. Wartha, M. Hornef, S. Normark, and B. H. Normark. 2005. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic. *Cell Microbiol.* **17**:1603–1615.
- Al-Salleeh, F., and T. M. Petro. 2008. Promoter analysis reveals critical roles for SMAD-3 and ATF-2 in expression of IL-23 p19 in macrophages. *J. Immunol.* **181**:4523–4533.
- Aujla, S. J., P. J. Dubin, and J. K. Kolls. 2007. Th17 cells and mucosal host defense. *Semin. Immunol.* **19**:377–382.
- Bai, B., K. Yamamoto, H. Sato, H. Sugiura, and T. Tanaka. 2007. Complex regulation of S100A8 by IL-17, dexamethasone, IL-4 and IL-13 in HaCat cells (human keratinocyte cell line). *J. Dermatol. Sci.* **47**:259–262.
- Beadling, C., and M. K. Slifka. 2006. IL-23 regulation of innate and adaptive immune responses by the related cytokines IL-12, and IL-27. *Arch. Immunol. Ther. Exp. (Warsz.)* **54**:15–24.
- Braun, R. K., C. Ferrick, P. Neubauer, M. Sjoding, A. Sterner-Kock, M. Kock, L. Putney, D. A. Ferrick, D. M. Hyde, and R. B. Love. 2008. IL-17 producing gammadelta T cells are required for a controlled inflammatory response after bleomycin-induced lung injury. *Inflammation* **31**:167–179.
- Carmody, R. J., Q. Ruan, H. C. Liou, and Y. H. Chen. 2007. Essential roles of c-Rel in TLR induced IL-23 p19 gene expression in dendritic cells. *J. Immunol.* **178**:186–191.
- Chen, T. C., Y. Y. Cheng, W. Z. Sun, and B. C. Shyu. 2008. Differential regulation of morphine antinociceptive effects by endogenous enkephalinergic system in the forebrain of mice. *Mol. Pain* **4**:41–59.
- Chung, D. R., D. L. Kasper, R. J. Panzo, T. Chitnis, M. J. Grusby, M. H. Sayegh, and A. O. Tzianabos. 2003. CD4+ T cells mediate abscess formation in intra-abdominal sepsis by an IL-17-dependent mechanism. *J. Immunol.* **170**:1958–1963.
- Dighe, S. V., P. A. Madia, S. Sirohi, and B. C. Yoburn. 2009. Continuous morphine produces more tolerance than intermittent or acute treatment. *Pharmacol. Biochem. Behav.* **92**:537–542.
- Dong, C. 2008. IL-23/IL-17 biology and therapeutic considerations. *J. Immunotoxicol.* **5**:43–46.
- Dubin, P. J., and J. K. Kolls. 2007. IL-23 mediates inflammatory responses to mucoid *Pseudomonas aeruginosa* lung infection in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **292**:L519–L528.
- Happel, K. I., P. J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L. J. Quinton, A. R. Odden, J. E. Shellito, G. J. Bagby, S. Nelson, and J. K. Kolls. 2005. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J. Exp. Med.* **202**:761–769.
- Happel, K. I., M. Zheng, E. Young, L. J. Quinton, E. Lockhart, A. J. Ramsay, J. E. Shellito, J. R. Schurr, G. J. Bagby, S. Nelson, and J. K. Kolls. 2003. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. *J. Immunol.* **170**:4432–4436.
- Iwamura, T., M. Yoneyama, K. Yamaguchi, W. Suhara, W. Mori, K. Shiota, Y. Okabe, H. Namiki, and T. Fujita. 2001. Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. *Genes Cells* **6**:375–388.
- Lemos, H. P., R. Grespan, S. M. Vieira, T. M. Cunha, W. A. Verri, Jr., K. S. Fernandes, F. O. Souto, I. B. McInnes, S. H. Ferreira, F. Y. Liew, and F. Q. Cunha. 2009. Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFN-gamma production. *Proc. Natl. Acad. Sci. USA* **106**:5954–5959.
- Loiario, M., C. Sette, G. Gallo, A. Ciacci, N. Fantò, D. Mastroianni, P. Carminati, and V. Ruggiero. 2005. Peptide-mediated interference of tir domain dimerization in myd88 inhibits IL-1-dependent activation of NF-κB. *J. Biol. Chem.* **16**:15809–15814.
- Lu, Y. J., J. Gross, D. Bogaert, A. Finn, L. Bagrade, Q. Zhang, J. K. Kolls, A. Srivastava, A. Lundgren, S. Forte, C. M. Thompson, K. F. Harney, P. W. Anderson, M. Lipsitch, and R. Malley. 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* **4**:e1000159.
- Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **223**:77–92.
- Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* **441**:231–234.
- Osikowicz, M., J. Mika, W. Makuch, and B. Przewlocka. 2008. Glutamate receptor ligands attenuate allodynia and hyperalgesia and potentiate morphine effects in a mouse model of neuropathic pain. *Pain* **139**:117–126.
- Paterson, G. K., and T. J. Mitchell. 2006. Innate immunity and the pneumococcus. *Microbiology* **152**(Pt 2):285–293.
- Raquil, M. A., N. Anceriz, P. Rouleau, and P. A. Tessier. 2008. Blockade of antimicrobial proteins S100A8 and S100A9 inhibits phagocyte migration to the alveoli in streptococcal pneumonia. *J. Immunol.* **180**:3366–3374.
- Roark, C. L., P. L. Simonian, A. P. Fontenot, W. K. Born, and R. L. O'Brien. 2008. Gammadelta T cells: an important source of IL-17. *Curr. Opin. Immunol.* **20**:353–357.
- Rook, E. J., J. M. van Ree, W. van den Brink, M. J. Hillebrand, A. D. Huiteima, V. M. Hendriks, and J. H. Beijnen. 2006. Pharmacokinetics and pharmacodynamics of high doses of pharmaceutically prepared heroin, by intravenous or by inhalation route in opioid-dependent patients. *Basic Clin. Pharmacol. Toxicol.* **98**:86–96.
- Roy, S., J. Wang, J. Kelschenbach, L. Koodie, and J. Martin. 2006. Modulation of immune function by morphine: implications for susceptibility to infection. *J. Neuroimmune Pharmacol.* **1**:77–89.
- Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J. Immunol.* **178**:4466–4472.
- Tiseo, P. J., H. T. Thaler, J. Lapin, C. E. Inturrisi, R. K. Portenoy, and K. M. Foley. 1995. Morphine-6-glucuronide concentrations and opioid-related side effects: a survey in cancer patients. *Pain* **61**:47–54.
- Vandal, K., P. Rouleau, A. Boivin, C. Ryckman, M. Talbot, and P. A. Tessier. 2003. Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. *J. Immunol.* **171**:2602–2609.
- Vila-Corcoles, A., O. Ochoa-Gondar, T. Rodriguez-Blanco, X. Raga-Luria, F. Gomez-Bertomeu, and EPIVAC Study Group. 2009. Epidemiology of community-acquired pneumonia in older adults: a population-based study. *Respir. Med.* **103**:309–316.
- Wang, J., R. A. Barke, R. Charboneau, and S. Roy. 2005. Morphine impairs host innate immune response and increases susceptibility to *Streptococcus pneumoniae* lung infection. *J. Immunol.* **174**:426–434.
- Wang, J., R. A. Barke, R. Charboneau, R. Schwendener, and S. Roy. 2008. Morphine induces defects in early response of alveolar macrophages to *Streptococcus pneumoniae* by modulating TLR9-NF-kappa B signaling. *J. Immunol.* **180**:3594–3600.
- Wang, J., R. A. Barke, J. Ma, R. Charboneau, and S. Roy. 2008. Opiate abuse, innate immunity, and bacterial infectious diseases. *Arch. Immunol. Ther. Exp. (Warsz.)* **56**:299–309.
- Yu, J. J., M. J. Ruddy, H. R. Conti, K. Boonantananasarn, and S. L. Gaffen. 2008. The interleukin-17 receptor plays a gender-dependent role in host protection against *Porphyromonas gingivalis*-induced periodontal bone loss. *Infect. Immun.* **76**:4206–4213.
- Zhang, Z., T. B. Clarke, and J. N. Weiser. 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J. Clin. Invest.* **119**:1899–1909.