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Morphine Induces Defects in Early Response of Alveolar Macrophages to *Streptococcus pneumoniae* by Modulating TLR9-NF-κB Signaling[1]

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Resident alveolar macrophages and respiratory epithelium constitutes the first line of defense against invading lung pneumococci. Results from our study showed that increased mortality and bacterial outgrowth and dissemination seen in morphine-treated mice were further exaggerated following depletion of alveolar macrophages with liposomal clodronate. Using an in vitro alveolar macrophages and lung epithelial cells infection model, we show significant release of MIP-2 from alveolar macrophages, but not from lung epithelial cells, following 4 h of exposure of cells to pneumococci infection. Morphine treatment reduced MIP-2 release in pneumococci stimulated alveolar macrophages. Furthermore, morphine treatment inhibited *Streptococcus pneumoniae*-induced NF-κB-dependent gene transcription in alveolar macrophages following 2 h of in vitro infection. *S. pneumoniae* infection resulted in a significant induction of NF-κB activity only in TLR9 stably transfected HEK 293 cells, but not in TLR2 and TLR4 transfected HEK 293 cells, and morphine treatment inhibited *S. pneumoniae*-induced NF-κB activity in these cells. Moreover, morphine treatment also decreased bacterial uptake and killing in alveolar macrophages. Taken together, these results suggest that morphine treatment impairs TLR9-NF-κB signaling and diminishes bacterial clearance following *S. pneumoniae* infection in resident macrophages during the early stages of infection, leading to a compromised innate immune response. ***The Journal of Immunology,*** **2008, 180: 3594–3600.**

Immunocompromise resulting from prolonged drug use contributes to the increased risk of respiratory tract infection (1). There is a 10-fold increase in the risk of community-acquired pneumonia in drug users (2). Bacterial pneumonia and invasive pneumococcal disease are strongly associated with HIV infection (3, 4). HIV-infected drug abusers have a higher incidence of pneumococcal pneumonia contributing to the morbidity and mortality observed in this population. HIV-seropositive injection drug users are at an ~4-fold greater risk of pneumococcal pneumonia than their HIV-seropositive nondrug-using counterparts (5, 6). These data suggest that drug abuse is a vital risk factor for pneumococcal infections. However, the mechanisms that underlay this increased susceptibility are unknown.

Alveolar macrophages (AMs)[4] play an important role in defense against airborne pathogens. During pneumococcal lung infection, pneumococci first encounter AMs. As the front line of cellular defense against pneumococcus, AMs phagocytose bacteria and coordinate the innate immune response to infection (7). AMs express various pattern recognition receptors, such as TLRs. TLRs play a crucial role against *Streptococcus pneumoniae* infection by initiating and activating both innate and adaptive immunity (8). TLR ligand engagement results in intracellular signal transduction, including activation of NF-κB, and synthesizing and secreting a wide array of proinflammatory cytokines such as interleukins-1, −6, and TNF-α, as well as chemokines such as MIP-2 and KC. Using these cell to cell signals, AMs initiate inflammatory responses and recruit activated neutrophils into the alveolar spaces (9).

Using a drug abuse and *S. pneumoniae* lung infection mouse model, our previous studies have shown that morphine treatment suppresses NF-κB gene transcription in resident lung tissue cells, which in turn, modulates the transcriptional regulation of MIP-2 and TNF-α. In addition, morphine treatment also results in a decrease in galectin-3 release into the bronchoalveolar lavage (BAL) in the early phase of infection. A combination of the decrease in MIP-2 and inflammatory cytokine synthesis and galectin-3 release in the early phase of infection leads to a reduction in neutrophil recruitment. This results in an increased pneumococcal bacterial burden within the lung tissue and the initiation of systemic disease (10). However, there are numerous gaps in our understanding of the mechanisms by which morphine affects NF-κB activation during *S. pneumoniae* infection. Our studies demonstrated that in the initial stage of infection, morphine decreased NF-κB activation in lung resident cells, but cell type within the lung tissue in which this decrease was observed was not delineated. There is no data showing how opioid receptors, activated by morphine, crosstalk with *S. pneumoniae* induced NF-κB signaling. Therefore, in this study, we have extended our previous findings by investigating the lung resident cell population that is involved in morphine modulation of innate immune response following pneumococcal infection. We

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[4] Abbreviations used in this paper: AMs, alveolar macrophages; BAL, bronchoalveolar lavage.

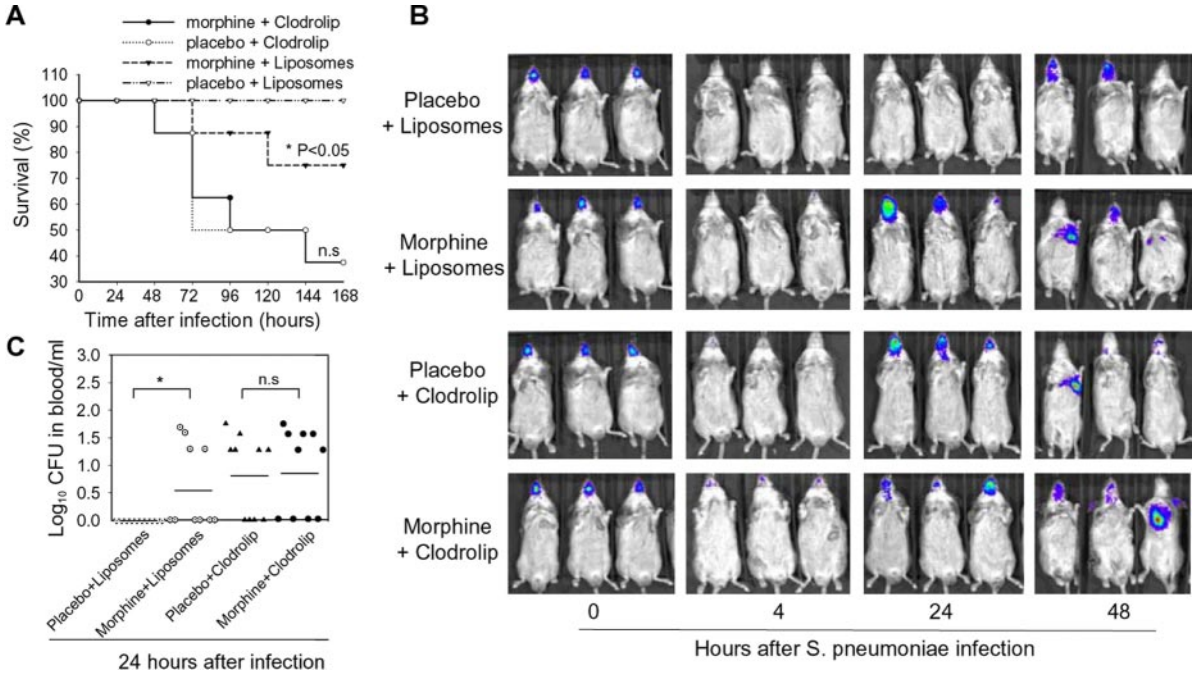

FIGURE 1. Morphine treatment increased susceptibility to pneumococcal infection and reduced survival in liposome-treated control mice. No difference in survival or in bacterial outgrowth and dissemination was observed between morphine- and placebo-treated groups in AM-depleted mice. *A*, Mice were infected intranasally with (10^7 CFU per mouse) *S. pneumoniae* (serotype 3). Mouse survival was assessed 7 days after infection ($n = 16$). $*$, $p < 0.05$, compared with placebo plus liposomes group; n.s., not significant, compared with placebo plus clodrolip group. *B*, Bacterial outgrowth in mice following intranasal inoculation with bioluminescent *S. pneumoniae* (serotype 3, Xen-10). Mice were imaged for 5 min at indicated time points. *C*, Bacteremia of mice. Blood samples (10 μl) were obtained from the tail vein at indicated time points. Results from individual mouse are shown ($n = 10$). $*$, $p < 0.05$; n.s., Not significant.

also determined the signaling pathways that underlie morphine's regulation of *S. pneumoniae*-induced NF-κB activity. Overall, our results reveal for the first time that morphine decreases bacterial clearance by resident AMs and impairs pneumococci-induced TLR9-NF-κB signaling. This leads to a decreased innate immune response at an early stage of infection before the entry of circulating inflammatory cells.

Materials and Methods

Experimental animal

Mice (B6129SF1) were obtained from The Jackson Laboratory. A maximum of four mice were housed per cage. Food and tap water were available ad libitum. The animal housing facilities were maintained on a 12-h light/dark cycle, with constant temperature (72 $\pm$ 1°F) and 50% humidity. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the University of Minnesota.

Pneumococcal pneumonia model and drug treatment protocol

A murine *pneumococcal pneumonia* model was used as previously described (10). In brief, mice were lightly anesthetized with isoflurane (Halocarbon Products) and inoculated with $\sim 10^7$ CFUs of *S. pneumoniae* serotype 3 (American Type Culture Collection) in 50 μl of PBS applied to the tip of the nose and involuntarily inhaled. Animals were implanted with either a 75-mg morphine pellet or placebo pellet (controls) 24 h before *S. pneumoniae* inoculation. The pellets were obtained from the National Institute on Drug Abuse.

Alveolar macrophage depletion

Each mouse intranasally received a dose of 1 mg liposome-encapsulated clodronate, or empty liposomes as control, 48 h before *S. pneumoniae* inoculation. Counting of AMs in BAL fluids from test animals indicated that this method depleted >94% of the AMs over a 5-day period (11).

In vivo monitoring of S. pneumoniae *infection in live mice*

Mice were lightly anesthetized with isoflurane and then inoculated intranasally with luciferase-tagged *S. pneumoniae* serotype 3 (Xen10; Xenogen). Whole body images were taken for 5 min using Xenogen's IVIS CCD camera system (Xenogen) at 0, 4, 24, and 48 h postinoculation. Total photon emission from selected and defined areas within the images of each mouse was quantified using the LivingImage software package from Xenogen (12).

BAL and neutrophil enumeration

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott Laboratories). BAL was obtained by instilling and collecting two 0.5-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 BAL were counted from each sample in a hemocytometer (Hausser Scientific). BAL neutrophil count was determined on cytospin preparations stained with a Diff-Quick staining kit (IMEB) (10).

Cells, in vitro morphine treatment, and cell infection

The following cells were used for the in vitro experiments:

MH-S (CRL-2019; American Type Culture Collection) is a murine AM cell line with a mature AM phenotype.

Mouse lung epithelial cell MLE-15 is an immortalized mouse lung epithelial cell line that maintains some morphological and functional characteristics of type II epithelial cells (13).

Stably transfected HEK 293 cells were used because they express fusion proteins consisting of CFP or YFP fused to TLRs at the C terminus. These cells were HEK293-pcDNA3, HEK293-TLR2-YFP, HEK293-TLR4/MD2-CFP, and HEK293-TLR9-YFP (14).

Cells were treated for 24 h in media containing vehicle (control) or variable concentrations of morphine (10 nM or 1 μM). Cells were infected with 10^6 CFU/ml *S. pneumoniae*, serotype 3, for various periods of time, depending on the experiment.

ELISA

MIP-2 in lung tissue of *S. pneumoniae* infected mice, and cell-culture of in vitro cell infection was quantified by using cytokine-specific ELISA kits (R&D Systems) according to the manufacturer's instructions.

Transfection and detection of luciferase reporter activity

Subconfluent mouse AMs, lung epithelial cells, and stably transfected HEK 293 cells (HEK293-pcDNA3, HEK293-TLR2, HEK293-TLR3, HEK293-TLR4/MD2, and HEK293-TLR9) were cotransfected with NF-κB-dependent luciferase reporter and pRL-TK (Promega) driving Renilla luciferase as an internal standard, using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. After 24 h of transfection, the cells were treated with morphine (10 nM or 1 μM) or vehicle for 24 h, then infected with *S. pneumoniae*. Lysates were harvested at 2 or 6 h after cell infection and assayed for firefly and Renilla luciferase activity using a dual luciferase reporter assay system (Promega) with a luminometer (Turner TD20/20; Promega). Values were reported as relative light units after correction for transfection efficiency by normalization with Renilla luciferase (15).

RNA interference for TLR9

Cells were transfected with either a negative control shRNA or Sure Silencing shRNA plasmid for mouse TLR9 according to the protocol from Superarray. Twenty four hours following transfection, TLR9 protein levels were determined by Western blot.

Western blot

Cells were washed with PBS and lysed in lysis buffer. TLR-9 was detected with polyclonal Ab to TLR-9 (Imgenex). Western blot assay of TLR9 was performed as previous described (15).

In vitro phagocytosis assay

A standard bacterial uptake and killing assay was used to evaluate the effect of treatment of AMs with morphine (10 nM and 1 μM). After incubation with morphine for 24 h at 37°C, AMs were exposed to *S. pneumoniae* for 1 h at 37°C (bacteria to macrophage ratio = 10:1). Unbound bacteria were removed using a PBS wash, repeated three times. To evaluate phagocytosis at this time point, 100 μg/ml gentamicin (Sigma-Aldrich) was added for 15 min at 37°C to kill extracellular bacteria. To study killing of ingested bacteria, the cells were incubated for another hour at 37°C. Samples at each stage were lysed and bacterial numbers measured by counting CFUs. The number of bacteria killed by the macrophages was determined by subtracting the number of viable CFUs counted from phagocytized CFUs.

Statistical analysis

Data were collected from three independent experiments and expressed as mean ± SEM. Significances were determined by Student's *t* test and two-way ANOVA analysis. Individual group comparisons were made by the two-tailed Student's *t* test. Statistical significance was accepted at $p < 0.05$. Survival was evaluated for differences using a log-rank test.

Results

Role of resident AMs in morphine treatment induced increased mortality, bacterial outgrowth, and dissemination

Resident AMs and respiratory epithelium constitute the first line of defense against invading pneumococci. To determine whether the AM is a critical cell population involved in morphine-induced impaired innate immunity, the effects of morphine and placebo treatments on mortality, bacterial outgrowth, and dissemination were investigated in pharmacologically depleted AMs and control mice. Morphine-treated mice were more susceptible to the pneumococcal infection and showed reduced survival in liposome-treated control mice. However, no difference in survival or in bacterial outgrowth and dissemination was observed between morphine- and placebo-treated groups in AM-depleted mice (Fig. 1*A–C*). The increased mortality, bacterial outgrowth, and dissemination seen in morphine-treated mice were further exaggerated by depletion of resident AMs with clodronate, suggesting that resident pulmonary AMs may be a critical cell population participating in morphine-induced compromise of innate immunity.

Role of AMs in morphine treatment induced delayed recruitment of neutrophils into the lungs

In previous studies, we have shown that chronic morphine treatment suppresses NF-κB gene transcription in resident cells of lung

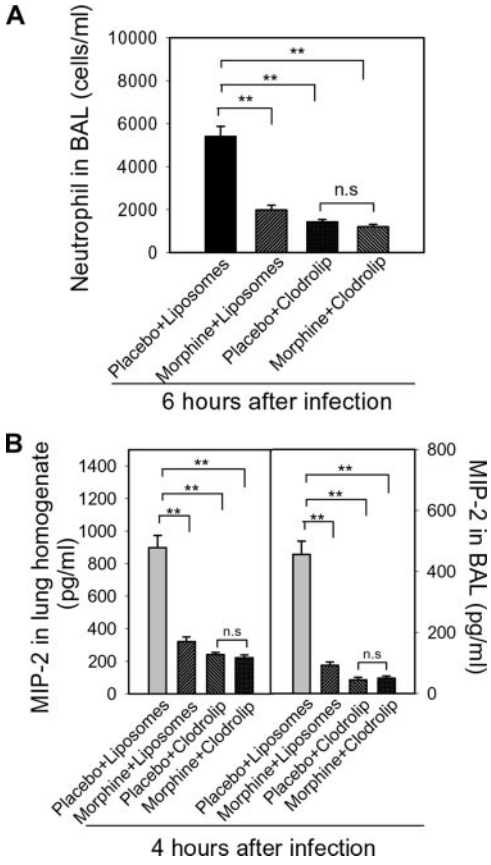

FIGURE 2. No difference in neutrophil migration and MIP-2 production was seen between morphine- and placebo-treated groups in AM-depleted mice. *A*, BAL fluid neutrophil numbers in control and treated mice 6 h after intranasal infection of *S. pneumoniae* are shown. *B*, MIP-2 concentrations are shown at 4 h after intranasal challenge with *S. pneumoniae.* Each value represents the mean ± SEM. Data are presented as the mean ± SEM. Mean values from three independent experiments are shown. n.s., Not significant; *, $p < 0.05$; **, $p < 0.01$.

tissue, which, in turn, modulates the transcriptional regulation of MIP-2 and TNF-α in the early phase of infection. This leads to a reduction in neutrophil recruitment, which results in an increase in bacterial burden within the lung tissue and the initiation of systemic disease (10). One possible explanation for this reduction in neutrophil recruitment is that morphine treatment impairs MIP-2 production in AMs at the early phase of infection. To address this possibility, we examined lung neutrophil number and MIP-2 concentration in control mice and mice with pharmacologically depleted AMs. Consistent with our previous studies, morphine treatment significantly decreased MIP-2 in both BAL fluid and lung homogenates. This correlated with a significant decrease in neutrophils yielded from lung BAL fluid during early step of infection in liposome-treated control mice. Interestingly, the decreased MIP-2 production and delayed neutrophil migration into the lung seen in morphine-treated mice were further reduced by AMs depletion (Fig. 2, *A* and *B*). These data suggest that AMs play an essential role in mediating a morphine-induced delay in neutrophil influx into the alveolar compartment in the early stage of infection.

Morphine reduced MIP-2 release and inhibited NF-κB-dependent gene transcription in pneumococci infected AMs

To further identify the lung resident cell population(s) responsible for morphine's actions, an in vitro AM and lung epithelial cell

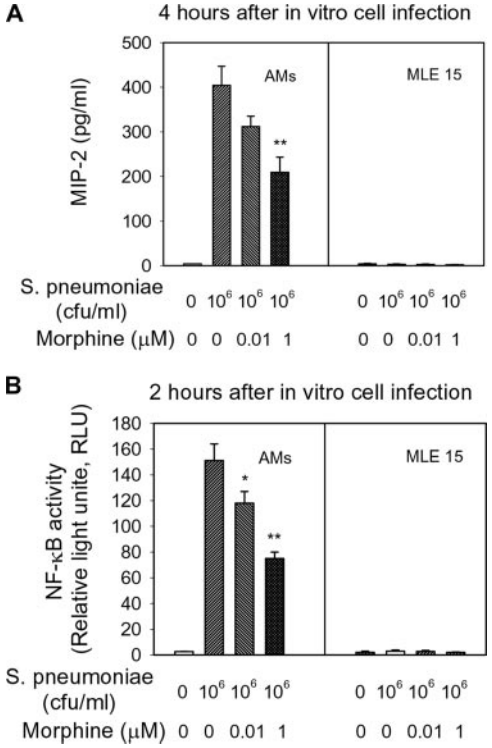

FIGURE 3. Effect of morphine treatment on pneumococci-induced MIP-2 release and NF-κB-dependent gene transcription in AMs and lung epithelial cells following *S. pneumoniae* infection. *A*, Mouse lung epithelial cells (MLE15) and AMs were treated with morphine (10 nM or 1 μM) or vehicle for 24 h, infected with the indicated concentrations of *S. pneumoniae* for 4 h, and MIP-2 concentration was measured in the supernatant by ELISA. *B*, Cells were treated with morphine (10 nM or 1 μM) or vehicle for 24 h before infection. Lysates were harvested after 2 h of cell infection. NF-κB-dependent gene transcription was evaluated by a dual luciferase reporter assay. **, $p < 0.01$ compared with the vehicle treatment group. Results are representative of three independent experiments.

infection model was used to test the effect of morphine treatment on MIP-2 production and NF-κB-dependent gene transcription following *S. pneumoniae* infection. The results presented in our previous study demonstrated that MIP-2 is a crucial factor in morphine increase susceptibility to *S. pneumoniae* infection (10). In this study, we found that significant MIP-2 release was detected in AMs after 4 h of cell infection. However, no detectable level of MIP-2 was observed in lung epithelial cells following *S. pneumoniae* infection. Morphine markedly reduced MIP-2 releases in pneumococci stimulated AMs after 4 h of infection. Similarly, NF-κB-dependent gene transcription was detected in AMs, but not in lung epithelial cells, following 2 h of cell infection. Morphine inhibited pneumococci-induced NF-κB-dependent gene transcription in AMs (Fig. 3, *A* and *B*). These data, along with the studies of AMs depletion, strongly suggest that AMs play a critical role in morphine induced impairment of innate immunity.

TLR9-mediated signaling, and not TLR2 or TLR4, plays a role in morphine inhibition of S. pneumoniae*-induced NF-κB activity in the early stage of infection*

To elucidate the mechanism by which morphine modulates AMs, we challenged stably transfected HEK 293 cells (HEK293-pcDNA3, HEK293-TLR2-YFP, HEK293-TLR4/MD2-CFP, and HEK293-TLR9-YFP) with *S. pneumoniae* (10^6 CFU/ml). As shown in Fig. 4, *S. pneumoniae* infection resulted in a significant

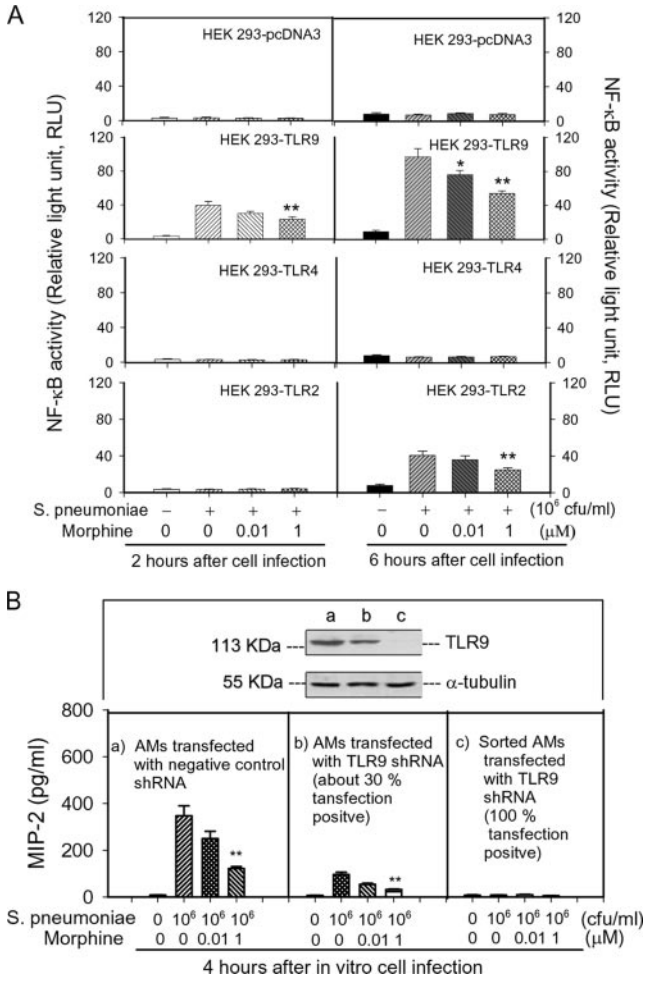

FIGURE 4. Effect of morphine on pneumococci-induced TLR9 dependent NF-κB activation. *A*, Stably transfected HEK 293 cells (HEK293-pcDNA3, HEK293-TLR2, HEK293-TLR4/MD2, and HEK293-TLR9) were cotransfected with NF-κB-dependent luciferase reporter, pRL-TK, and μ-opioid receptors (MOR-EGFP) plasmid DNA or vector plasmid DNA. Cells were treated with morphine (10 nM or 1 μM) or vehicle for 24 h. Lysates were harvested after 2 and 6 h of cell infection with *S. pneumoniae*. NF-κB-dependent gene transcription was evaluated by a dual luciferase reporter assay. *B*, AMs were transfected with shRNA for TLR9 as described, treated with either morphine (10 nM or 1 μM) or vehicle for 24 h, and infected with *S. pneumoniae* for 24 h. ELISAs were performed on the cell supernatant to assess the protein levels of MIP-2. Concentrations were extrapolated from standard curves, and the data are presented as mean concentration ± SEM. **, $p < 0.01$; *, $p < 0.05$ compared with the vehicle control group; $n = 6$. Results are representative of three independent experiments.

induction of NF-κB activity in HEK 293-TLR9 cells, but not in HEK293-TLR2 and HEK293-TLR4, following 2 h of cell infection. After 6 h of cell infection, *S. pneumoniae* infection led to significant induction of NF-κB activity in both HEK 293-TLR9 and HEK 293-TLR2 cells. Morphine treatment inhibited *S. pneumoniae*-induced NF-κB activity in HEK 293-TLR9 cells following 2 h of cell infection (Fig. 4*A*). These data indicated that morphine treatment impaired innate immune defenses against *S. pneumoniae* by modulating TLR9-NF-κB signaling in the early stage of infection. However, TLR-2 signaling may also participate in morphine's action at a later stage of infection because morphine also inhibits TLR2-induced NF-κB activation following 6 h of cell infection.

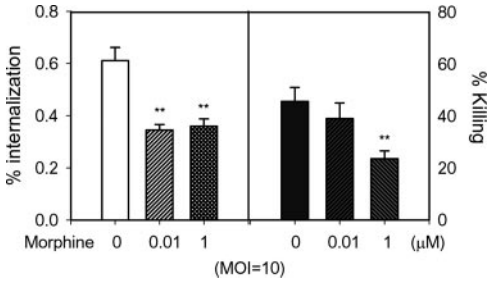

FIGURE 5. Morphine effects on interaction of macrophages with *S. penumoniae*. Morphine treatment caused decreased internalization. Macrophages were challenged at multiplicity of infection (MOI) = 10 for 60 min. Morphine decreased total killing. Macrophage killing was measured with an in vitro gentamicin protection assay. Viable intracellular bacteria were quantified by lysis of macrophages and subsequent plating on blood agar plates. All values are mean ± SEM for three separate experiments, **, $p < 0.01$; *, $p < 0.05$ compared with the vehicle control group; $n = 6$. Results are representative of three independent experiments.

To further confirm that morphine modulated TLR9-NF-κB signaling in AMs, MIP-2 production following *S. pneumoniae* infection was investigated using AMs transfected with TLR9-GFP shRNA plasmid DNA. A high level of TLR9 expression was seen in negative control shRNA-vector transfected AMs. Morphine treatment decreased MIP-2 production following 4 h of infection. A significant lower level of MIP-2 was shown in TLR9 shRNA plasmid DNA transfected AMs (Fig. 4*B*). A noninfected baseline level of MIP-2 was detected in TLR9 shRNA plasmid DNA transfection positive AMs (enriched by FACS cell sorting for GFP-expressing cells) (Fig. 4*C*). These results further support the concept that TLR9-NF-κB signaling acts at an early stage in host defense against pneumococcal infection and morphine treatment impairs this signaling.

Effect of morphine treatment on the interaction of AMs with S. pneumoniae

As the first line of cellular defense against pneumococcus, AMs phagocytose bacteria and coordinate the innate immune response to infection. Impairment of AMs interaction with *S. pneumoniae* may be one of the mechanisms by which morphine increased host susceptibility to pneumococcal infection. In this in vitro study, we measured the bacterial internalization and killing in AMs. Compared with the control group treated with vehicle, internalization of *S. pneumoniae* was decreased in both the 10 nM and 1 μM dose of morphine-treated AMs; however, the rate of killing of internalized bacteria was decreased in only high dose morphine-treated AMs (Fig. 5, *A* and *B*). Taken together, all above shown data suggest that morphine-induced modulation of the innate immune response is mediated by both diminishing bacterial clearance and impairing TLR9-NF-κB signaling, which are host defense mechanisms that occur at the early stage following pneumococcal infection.

Discussion

In vivo pharmacologic and in vitro cell infection approaches were used to investigate the role of AMs in morphine modulation of innate immunity in response to *S. pneumoniae* infection. Our studies support the concept that resident AMs are a critical cell population in the lung, participating in bacterial clearance and coordinating the innate immune response at an early stage of infection. Morphine treatment impaired the initiation of innate immune defenses, following *S. pneumoniae* infection, by inactivating TLR9-NF-κB signaling in AMs.

Early events that take place in the first hours following *S. pneumoniae* infection are crucial to determine whether the infection will be controlled and cleared or progress to a diseased state (16). Our previous studies showed that morphine treatment resulted in a significant suppression of MIP-2 and KC during the early phase of infection. This suppression correlated with reduced neutrophil recruitment, increased bacterial outgrowth and dissemination, and mortality observed in morphine-treated mice (10). Our results suggest that morphine treatment delays the initiation of the innate immune response to *S. pneumoniae*. The focus of this study was to determine the role of the TLRs-NF-κB signaling in morphine-induced MIP-2 production observed at the early stage following *S. pneumoniae* infection (2 and 4 h after infection).

Resident AMs are generally considered to play a prominent role in the initiation and regulation of innate immunity due to their diverse repertoire of functions (17). In addition to phagocytosis and microbiocidal activities, resident AMs secrete a variety of chemokines and cytokines that directly or indirectly result in inflammatory cell recruitment to the lung and in the up-regulation of local host defense mechanisms (18). Depletion studies have identified AMs as major contributors to pulmonary inflammation and cytokine production, including TNF-α and IL-1β (19–22). Our previous study showed that morphine decreased chemokine (MIP-2 and KC) and cytokine (TNF-α, IL-1, and IL-6) production in lung at an early stage of infection, prior inflammatory cell recruitment to the lung, suggesting that the morphine's effect was mediated by resident lung cells, including AMs and epithelial cells (10). In this study, we extended our previous observations to investigate the cell population responsible for morphine's action. Our data show that depletion of AMs further exaggerated morphine-induced increase in mortality and dissemination following *S. pneumoniae* infection. In an in vitro cell infection model, diminished NF-κB activation and MIP-2 production induced by morphine treatment were only observed in *S. pneumoniae* infected AMs, not in pulmonary epithelial cells at the early stage of infection. It is clear from the results presented in the current study that morphine treatment impaired the initial inflammatory response and phagocytocytic function of resident macrophage before the infiltration of inflammatory cells. This led to an increased susceptibility to *S. pneumoniae* lung infection.

The NF-κB activation in macrophages is an important signal transduction pathway in the development of the lung innate immune response (23, 24). A series of data suggests that AMs play a critical sentinel role in mediating NF-κB activation in the lung (25). Depletion of pulmonary macrophages by intratracheal administration of liposome-encapsulated dichloromethylene diphosphonate attenuates activation of lung NF-κB in whole lung tissue in mice that are treated with endotoxin. Suppression of neutrophilic lung inflammation by macrophage depletion is associated with substantially reduced BAL levels of inflammatory cytokines, chemokine, and adhesion molecules in response to treatment with endotoxin (26). These data suggest that macrophages play a role in initiating an NF-κB-mediated response to endotoxemia. But the molecular mechanisms by which macrophage is activated in response to gram-positive bacteria *S. pneumoniae* have not been defined. Our in vitro cell infection study showed that NF-κB activity in response to *S. pneumoniae* infection is seen only in resident AMs at the early stage of infection (2 h after infection). Depletion of lung macrophages in vivo reduced neutrophils recruitment to lungs infected with *S. pnemoniae* in the early phase of infection (6 h after infection). These data suggest that resident AMs play a critical role in initiating an NF-κB-mediated response to *S. pneumoniae*. Morphine treatment inhibited initiating a NF-κB

activation in response to *S. pneumoniae* in AMs, leading to impaired initiation of innate immune response to infection.

Macrophages recognize *S. pneumoniae* by interacting with members of the TLR family. The ability of *S. pneumoniae* to activate TLR2 is well described and involves recognition of the cell wall components peptidoglycan and lipoteichoic acid (27, 28). In addition, there is evidence that pneumolysin is able to stimulate cells through TLR4, although this has not been found in all studies (29, 30). *S. pneumoniae* also activates TLR9, which recognizes unmethylated CpG DNA. But *S. pneumoniae*-induced NF-κB activation through TLR-9 is dependent on the viability of the bacteria, because only live *S. pneumoniae* are recognized by TLR-9. This suggests that infection with live pathogens may lead to activation of pattern recognition receptors not targeted by inactivated bacteria (31). Therefore, in the in vitro cell infection study, we used live *S. pneumoniae*. Interestingly, a recent publication showed that TLR9 plays a protective role in the lungs at an early stage of infection before the entry of circulating inflammatory cells. Resident AMs isolated from TLR9-deficient mice were defective in bacterial uptake, suggesting that the increased susceptibility to pneumococcal infection was due to a deficient clearance of bacteria in the lower respiratory tract in the early stage of infection. If this defect is due to a defective response to CpG containing bacterial DNA or to an inherited phagocytosis defect of $TLR9^{-/-}$ macrophages remains to be elucidated (32). In agreement with this group's study, using TLRs transfection cells and TLR9 shRNA transfected AMs, we found that NF-κB activity and MIP-2 production in response to *S. pneumoniae* was through TLR9 signaling at the very early stage of infection. Morphine treatment impaired the early TLR9-NF-κB signaling.

Previous studies showed that morphine inhibits phagocytosis by murine peritoneal macrophages through μ and δ opioid receptors by alterations in the cAMP pathway (33–36). In this work, we found that morphine impaired AM-mediated clearance of *S. pneumoniae*. Our unpublished data show that morphine treatment of macrophages inhibits actin polymerization, phagosome formation, and phagolysosomal fusion through a mechanism that involves Rac-GTP. Thus, our hypothesis is that in the *S. pneumoniae* infection animal model, failure to initiate phagolysosomal fusion may be a mechanism by which activation of TLR9 is blunted following morphine treatment. In future studies, we will investigate whether failure to initiate phagolysosomal fusion events may be a potential mechanism by which morphine treatment modulates TLR9 signaling.

The interplay between opioid abuse and the modulation of immune response to *S. pneumoniae* infection appear to be highly complex. Understanding the cellular and molecular mechanisms by which morphine impairs the innate immune response to pneumococcal infection is important to gain insights into developing therapeutic intervention to control pneumococcal infection in the drug abuse population. Our studies indicate that resident AMs are an essential cell population that participates in morphine's effect. During early stage of infection, two events, inhibition of TLR9-NF-κB signaling and decreased bacterial clearance, may play a role in morphine-induced delay in the initiation of innate immune response to *S. pneumoniae* infection. TLR9-NF-κB signaling may be a promising therapeutic target in *S. pneumoniae* infected drug abuser.

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Disclosures

The authors have no financial conflict of interest.

References

1. Boschini, A., C. Smacchia, M. Di Fine, A. Schiesari, P. Ballarini, M. Arlotti, C. Gabrielli, G. Castellani, M. Genova, P. Pantani, et al. 1996. Community-acquired pneumonia in a cohort of former injection drug users with and without human immunodeficiency virus infection: incidence, etiologies, and clinical aspects. *Clin. Infect. Dis.* 23: 107–113.
2. Tumbarello, M., E. Tacconelli, K. de Gaetano, F. Ardito, T. Pirronti, R. Cauda, and L. Ortona. 1998. Bacterial pneumonia in HIV-infected patients: analysis of risk factors and prognostic indicators. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 18: 39–45.
3. Feldman, C. 2005. Pneumonia associated with HIV infection. *Curr. Opin. Infect. Dis.* 18: 165–170.
4. Klugman, K. P., S. A. Madhi, and C. Feldman. 2007. HIV and pneumococcal disease. *Curr. Opin. Infect. Dis.* 20: 11–15.
5. Hirshtick, R., J. Glassroth, and M. Jordan. 1995. Bacterial pneumonia in persons infected with HIV. *N. Engl. J. Med.* 333: 845–851.
6. Selwyn, P., A. Feingold, D. Hartel, E. Schoenbaum, M. Alderman, R. Klein, and G. Friedland. 1988. Increased risk of bacterial pneumonia in HIV-infected IDU without AIDS. *AIDS* 2: 267–272.
7. Kadioglu, A., and P. W. Andrew. 2004. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol.* 25: 143–149.
8. Albiger, B., S. Dahlberg, B. Henriques-Normark, and S. Normark. 2007. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. *J. Intern. Med.* 261: 511–528.
9. Schmeck, B., S. Huber, K. Moog, J. Zahlten, A. C. Hocke, B. Opitz, S. Hammerschmidt, T. J. Mitchell, M. Kracht, S. Rosseau, et al. 2006. *S. Pneumococci*-induced TLR- and Rac1-dependent NF-κB-recruitment to the IL-8 promoter in lung epithelial cells. *Am. J. Physiol.* 290: L730–L737.
10. Wang, J., R. A. Barke, R. Charboneau, and S. Roy. 2005. Morphine impairs host innate immune response and increases susceptibility to *Streptococcus pn*eumoniae lung infection. *J. Immunol.* 174: 426–434.
11. Beck-Schimmer, B., R. Schwendener, T. Pasch, L. Reyes, C. Booy, and R. C. Schimmer. 2005. Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury. *Respir. Res.* 6: 61–76.
12. Francis, K. P., J. Yu, C. Bellinger-Kawahara, D. Joh, M. J. Hawkinson, G. Xiao, T. F. Purchio, M. G. Caparon, M. Lipsitch, and P. R. Contag. 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive lux transposon. *Infect. Immun.* 69: 3350–3358.
13. Bruno, M. D., T. R. Korfhagen, C. Liu, E. E. Morrisey, and J. A. Whitsett. 2000. GATA-6 activates transcription of surfactant protein A. *J. Biol. Chem.* 275: 1043–1049.
14. Hise, A. G., K. Daehnel, I. Gillette-Ferguson, E. Cho, H. F. McGarry, M. J. Taylor, D. T. Golenbock, K. A. Fitzgerald, J. W. Kazura, and E. Pearlman. 2007. Innate immune responses to endosymbiotic Wolbachia bacteria in *Brugia malayi* and *Onchocerca volvulus* are dependent on TLR2, TLR6, MyD88, and Mal, but not TLR4, TRIF, or TRAM. *J. Immunol.* 178: 1068–1076.
15. Wang, J., R. A. Barke, and S. Roy. 2007. Transcriptional and epigenetic regulation of interleukin-2 gene in activated T cells by morphine. *J. Biol. Chem.* 282: 7164–7171.
16. Dallaire, F., N. Ouellet, Y. Bergeron, V. Turmel, M. C. Gauthier, M. Simard, and M. G. Bergeron. 2001. Microbiological and inflammatory factors associated with the development of pneumococcal pneumonia. *J. Infect. Dis.* 184: 292–300.
17. Kooguchi, K., S. Hashimoto, A. Kobayashi, Y. Kitamura, I. Kudoh, J. Wiener-Kronish, and T. Sawa. 1998. Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect. Immun.* 66: 3164–3149.
18. Lomas-Neira, J. L., C. S. Chung, D. E. Wesche, M. Perl, and A. Ayala. 2005. In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 versus KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury. *J. Leukocyte Biol.* 77: 846–853.
19. Lomas-Neira, J., C. S. Chung, M. Perl, S. Gregory, W. Biffl, and A. Ayala. 2006. Role of alveolar macrophage and migrating neutrophils in hemorrhage-induced priming for ALI subsequent to septic challenge. *Am. J. Physiol.* 290: L51–L58.
20. Broug-Holub, E., G. B. Toews, J. F. van Iwaarden, R. M. Strieter, S. L. Kunkel, R. Paine, III, and T. J. Standiford. 1997. Alveolar macrophages are required for protective pulmonary defenses in murine Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect. Immun.* 65: 1139–1146.
21. Hashimoto, S., J. F. Pittet, K. Hong, H. Folkesson, G. Bagby, L. Kobzik, C. Frevert, K. Watanabe, S. Tsurufuji, and J. Wiener-Kronish. 1996. Depletion of alveolar macrophages decreases neutrophil chemotaxis to *Pseudomonas* airspace infections. *Am. J. Physiol.* 270: L819–L828.
22. Lentsch, A. B., B. J. Czermak, N. M. Bless, N. Van Rooijen, and P. A. Ward. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-κB. *Am. J. Respir. Cell Mol. Biol.* 20: 692–698.
23. Park, G. Y., and J. W. Christman. 2006. Nuclear factor κB is a promising therapeutic target in inflammatory lung disease. *Curr. Drug Targets* 7: 661–668.

24. Hippenstiel, S., B. Opitz, B. Schmeck, and N. Suttorp. 2006. Lung epithelium as a sentinel and effector system in pneumonia: molecular mechanisms of pathogen recognition and signal transduction. *Respir. Res.* 7: 97–105.
25. Lentsch, A. B., B. J. Czermak, N. M. Bless, N. Van Rooijen, and P. A. Ward. 1999. Essential role of alveolar macrophages in intrapulmonary activation of NF-κB. *Am. J. Respir. Cell Mol. Biol.* 20: 692–698.
26. Conron, M., E. Andreakos, P. Pantelidis, C. Smith, H. L. Beynon, R. M. Dubois, and B. M. Foxwell. 2002. Nuclear factor-κB activation in alveolar macrophages requires IκB kinase-β, but not nuclear factor-κB inducing kinase. *Am. J. Respir. Crit. Care Med.* 165: 996–1004.
27. Schroder, N. W., S. Morath, C. Alexander, L. Hamann, T. Hartung, U. Zahringer, U. B. Gobel, J. R. Weber, and R. R. Schumann. 2003. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* 278: 15587–15594.
28. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol.* 163: 1–5.
29. Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci. USA* 100: 1966–1971.
30. Srivastava, A., P. Henneke, A. Visintin, S. C. Morse, V. Martin, C. Watkins, J. C. Paton, M. R. Wessels, D. T. Golenbock, and R. Malley. 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect. Immun.* 73: 6479–6487.
31. Mogensen, T. H., S. R. Paludan, M. Kilian, and L. Ostergaard. 2006. Live *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J. Leukocyte Biol.* 80: 267–277.
32. Albiger, B., S. Dahlberg, A. Sandgren, F. Wartha, K. Beiter, H. Katsuragi, S. Akira, S. Normark, and B. Henriques-Normark. 2007. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cell. Microbiol.* 9: 633–644.
33. Lázaro, M. I., N. Tomassini, I. González, and F. L. Renaud. 2000. Reversibility of morphine effects on phagocytosis by murine macrophages. *Drug Alcohol Depend.* 58: 159–164.
34. Tomei, E. Z., and F. L. Renaud. 1997. Effect of morphine on Fc-mediated phagocytosis by murine macrophages in vitro. *J. Neuroimmunol.* 74: 111–116.
35. Lugo-Chinchilla, A. M., D. Báez, M. Vélez, C. Ildefonso, and F. L. Renaud. 2006. Altered subcellular signaling in murine peritoneal macrophages upon chronic morphine exposure. *J. Neuroimmunol.* 176: 86–94.
36. Tomassini, N., F. Renaud, S. Roy, and H. H. Loh. 2004. Morphine inhibits Fc-mediated phagocytosis through μ and δ opioid receptors. *J. Neuroimmunol.* 147: 131–133.