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

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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.


Immuno-compromise 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 non-drug-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) 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...
also determined the signaling pathways that underlie morphine’s regulation of \( S. \) pneumoniae-induced NF-\( \kappa \)B activity. Overall, our results reveal for the first time that morphine decreases bacterial clearance by resident AMs and impairs pneumococci-induced TLR9-NF-\( \kappa \)B 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 ± 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 \( S. \) pneumoniae model was used as previously described (10). In brief, mice were lightly anesthetized with isoflurane (Halo-\textregistered\ carbon Products) and inoculated with \( \approx 10^7 \) CFUs of \( S. \) pneumoniae serotype 3 (American Type Culture Collection) in 50 \( \mu \)l 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 clodrolip, 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). 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).

\( \text{BAL and neutrophil enumeration} \)

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-1909; 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 transected 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 \( \mu \)M). Cells were infected with \( 10^8 \) 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-pDNA3, 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. 1A–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 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, in vitro AM and lung epithelial cell
infection model was used to test the effect of morphine treatment on MIP-2 production and NF-kB-dependent gene transcription 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. NF-kB-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.

FIGURE 3. Effect of morphine treatment on pneumococci-induced MIP-2 release and NF-kB-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. NF-kB-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.

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.

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 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. 4A). 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.
S. pneumoniae measured the bacterial internalization and killing in AMs. Com-
susceptibility to pneumococcal infection. In this in vitro study, we may be one of the mechanisms by which morphine increased host
defense against pneumococcal infection and morphine treatment
impairs the 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. Com-
pared 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. 5A 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 stud-
ies support the concept that resident AMs are a critical cell pop-
ulation in the lung, participating in bacterial clearance and co-
dinating the innate immune response at an early stage of infection.
Morphine treatment impaired the initiation of innate immune de-
fenses, following S. pneumoniae infection, by inactivating TLR9-
NF-κB signaling in AMs.

Early events that take place in the first hours following S. pneu-
moniae 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 re-
cruitment, increased bacterial outgrowth and dissemination, and
mortality observed in morphine-treated mice (10). Our results sug-
gest 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-in-
duced 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 che-
mokines and cytokines that directly or indirectly result in inflam-
mmatory cell recruitment to the lung and in the up-regulation of
local host defense mechanisms (18). Depletion studies have iden-
tified 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 re-
cruitment 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 in-
vestigate the cell population responsible for morphine’s action.
Our data show that depletion of AMs further exaggerated mor-
phine-induced increase in mortality and dissemination following S.
pneumoniae infection. In an in vitro cell infection model, dimin-
ished NF-κB activation and MIP-2 production induced by mor-
phine treatment were only observed in S. pneumoniae infected
AMs, not in pulmonary epithelial cells at the early stage of infec-
tion. It is clear from the results presented in the current study that
morphine treatment impaired the initial inflammatory response and
phagocytotic function of resident macrophage before the inflit-
tration of inflammatory cells. This led to an increased susceptibil-
ity 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 admin-
istration of liposome-encapsulated dichloromethylene diphos-
phonate attenuates activation of lung NF-κB in whole lung tissue
in mice that are treated with endotoxin. Suppression of neutro-
philic lung inflammation by macrophage depletion is associated
with substantially reduced BAL levels of inflammatory cytokines,
chemokines, 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 re-
sponse to gram-positive bacteria S. pneumoniae have not been de-
efined. 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. pneumoniae 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

FIGURE 5. Morphine effects on interaction of macrophages with S.
pneumoniae. Morphine treatment caused decreased internalization. Mac-
rophages 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.
6. Results are representative of three independent experiments.
activation in response to \textit{S. pneumoniae} in AMs, leading to impaired initiation of innate immune response to infection.

Macrophages recognize \textit{S. pneumoniae} by interacting with members of the TLR family. The ability of \textit{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 polyinosinyl is able to stimulate cells through TLR4, although this has not been found in all studies (29, 30). \textit{S. pneumoniae} also activates TLR9, which recognizes unmethylated CpG DNA. But \textit{S. pneumoniae}-induced NF-\textit{kB} activation through TLR9 is dependent on the viability of the bacteria, because only live \textit{S. pneumoniae} are recognized by TLR9. 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 \textit{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, this would be consistent with that group’s study, using TLRs transfection cells and amplifying with this group’s study, using TLRs transfection cells and amplifying.


