TLR4-dependent GM-CSF protects against lung injury in Gram-negative bacterial pneumonia

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TLR4-dependent GM-CSF protects against lung injury in Gram-negative bacterial pneumonia. Am J Physiol Lung Cell Mol Physiol 302: L447–L454, 2012. First published December 9, 2011; doi:10.1152/ajplung.00415.2010.—Toll-like receptors (TLRs) are required for protective host defense against bacterial pathogens. However, the role of TLRs in regulating lung injury during Gram-negative bacterial pneumonia has not been thoroughly investigated. In this study, experiments were performed to evaluate the role of TLR4 in pulmonary responses against Klebsiella pneumoniae (Kp). Compared with wild-type (WT) (Balb/c) mice, mice with defective TLR4 signaling (TLR4-/- mice) had substantially higher lung bacterial colony-forming units after intratracheal challenge with Kp, which was associated with considerably greater lung permeability and lung cell death. Reduced expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA and protein was noted in lungs and bronchoalveolar lavage fluid of TLR4-/- mice postintratracheal Kp compared with WT mice, and primary alveolar epithelial cells (AEC) harvested from TLR4-/- mice produced significantly less GM-CSF in vitro in response to heat-killed Kp compared with WT AEC. TLR4-/- AEC underwent significantly more apoptosis in response to heat-killed Kp in vitro, and treatment with GM-CSF protected these cells from apoptosis in response to Kp. Finally, intratracheal administration of GM-CSF in TLR4-/- mice significantly decreased albumin leak, lung cell apoptosis, and bacteremia in Kp-infected mice. Based on these observations, we conclude that TLR4 plays a protective role on lung epithelium during Gram-negative bacterial pneumonia, an effect that is partially mediated by GM-CSF.

Klebsiella; Toll receptors; acute lung injury; growth factors

ACUTE LUNG INJURY (ALI) and acute respiratory distress syndrome (ARDS) continue to be associated with high morbidity and mortality. Pneumonia is a leading cause of ALI/ARDS and associated mortality in immunocompetent individuals in the United States. Klebsiella pneumoniae (Kp) is a Gram-negative bacteria that is a well-described cause of both community-acquired and hospital-acquired pneumonia and has a propensity to induce substantial tissue necrosis and ALI (11, 25).

Toll receptors are pattern recognition receptors that are activated by both pathogen-associated molecular patterns and endogenous danger-associated molecular patterns. Toll-like receptor (TLR) 4 is activated by lipopolysaccharide (LPS), resulting in stimulation of protective innate immune responses. In murine models of Gram-negative bacterial pneumonia, TLR4 mediates the elaboration of cytokines and chemokines that facilitate early recruitment and activation of polymorphonuclear leukocytes (PMN), responses that are required for local bacterial clearance and prevention of systemic dissemination (5, 23). TLR4 is also required for maintenance of alveolar epithelial integrity in response to noninfectious insults, including bleomycin and hyperoxic exposure (16, 26). The mechanism of by which TLR4 activates prosurvival responses in alveolar epithelium has not been clearly defined but is believed to be partially mediated by activation of nuclear factor-κB (NF-κB)-driven anti-apoptotic pathways (16).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a product of many cells, including alveolar epithelial cells (AEC). This pleiotropic growth factor serves as an important activating and differentiation factor for alveolar macrophages, effects required for effective pulmonary innate immunity and surfactant homeostasis. Impaired immunity against both bacteria (Gram-negative) and fungi has been observed in GM-CSF-deficient mice (2, 3, 7). Finally, there is compelling evidence that GM-CSF exerts cytoprotective properties on epithelial surfaces, particularly the alveolar epithelium. GM-CSF is an AEC mitogen and can protect alveolar epithelial integrity against a variety of insults, including hyperoxia, endotoxin, and bleomycin-induced injury (1, 18, 20). Pretreatment with GM-CSF has recently been shown to substantially reduce lung injury and improve survival in a murine model of H1N1 influenza pneumonia (14). Consequently, recombinant human CSF has been used in the clinical treatment of infections, lung injury, and wound healing.

In the present study, we hypothesized that TLR4 is important for preventing lung injury and alveolar cell apoptosis in bacterial pneumonia, an effect that may be partially attributable to regulation of GM-CSF. We found that mice with defective TLR4 signaling (13) (TLR4-/- mice) displayed markedly impaired bacterial clearance, severe ALI, and lung epithelial cell death after challenge with Kp intratracheally compared with wild-type (WT) mice. Enhanced lung injury responses in TLR4-/- mice were associated with reduced pulmonary GM-CSF expression, as well as impaired GM-CSF production by AECs recovered from TLR4-/- mice when incubated with Kp in vitro. Intratracheal treatment with GM-CSF protected TLR4-/- mice against lung injury and limited bacterial dissemination, an effect that was disproportionate to changes in lung bacterial clearance. Our findings suggest that TLR4 is required for both innate antibacterial defense mechanisms and for maintaining the epithelial barrier in acute Gram-negative pneumonia, which is partly due to production of GM-CSF.

METHODS

Mice. Six- to eight-week-old TLR4-/- mice and strain-matched WT Balb/c mice (purchased from Jackson Laboratory) maintained at...
the University of Michigan Unit for Laboratory Animal Medicine were used. All animals are treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Reagents. Recombinant mouse GM-CSF was obtained from R&D Systems (Minneapolis, MN).

Intratracheal inoculation. Mice were anesthetized with an intraperitoneal ketamine and xylazine mixture. For intratracheal inoculation of Klebsiella, the trachea was exposed, and 30 μl of inoculum was administered via a sterile 26-gauge needle. The skin incision was closed using surgical staples.

Murine AEC isolation. Primary type 2 AEC from WT and mutant mice were isolated as previously published (19). Briefly, after mice were heparinized and killed, they were exsanguinated, and lungs were perfused with saline. The lungs were filled with Dispase (1–2 ml; Worthington), followed by 0.45 ml of low-melting-point agarose and placed in 2 ml of Dispase. Lungs were incubated at 24°C for 45 min and then lung tissue was teased away from the airways and minced in DMEM with 0.1% DNase. Lung minces were filtered through 100-, 43-, and 15-mm nylon mesh filters. Cells were collected by centrifugation and then incubated with anti-CD32 and anti-CD45 antibodies. Cells are then incubated with streptavidin-coated magnetic particles, and positive bone marrow-derived cells were collected on a magnetic column. The negative cells were collected, and mesenchymal cells were removed by adherence purification overnight. We have shown that these type II cells are 96% pure by intermediate filament staining (6, 8).

Whole lung homogenization for colony-forming units determination. At designated time points, the mice were killed by CO₂ inhalation. Before lung removal, the pulmonary vasculature was perfused by infusing 1 ml of PBS containing 5 mM EDTA in the right ventricle. Whole lungs were removed, taking care to dissect away lymph nodes. The lungs were then homogenized in 1 ml of PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, IN). Homogenates were then serially diluted 1:5 in PBS and plated on blood agar to determine lung colony-forming units (CFU).

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was performed for collection of bronchoalveolar lavage fluid (BALF) as previously described (9). Briefly, the trachea was exposed and instilled using a 1.7-mm outer diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1-ml aliquots. A total of 3 ml PBS were instilled per mouse, with 90% of lavage fluid retrieved.

Real-time quantitative RT-PCR. Measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystem, Foster City, CA) as previously described (9). Briefly, total cellular RNA from the frozen lungs were isolated, reversed transcribed into cDNA, and then amplified using specific primers for GM-CSF, with β-actin serving as a control. Specific thermal cycling parameters used with the TaqMan One-Step RT-PCR Master Mix Reagents kit included 30 min at 48°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Relative quantitation of cytokine mRNA levels was plotted as the fold change compared with untreated control cells or whole lung. All experiments were performed in duplicate.

Murine ELISA, GM-CSF levels were quantitated using a modification of a double-ligand method as previously described (12). The enzyme-linked immunosorbent assay (ELISA) method used consistently detected murine cytokine concentrations above 20–50 pg/ml. The ELISAs did not cross-react with other cytokines tested.

Murine ELISA for albumin measurement. Albumin (Albunin Quantification Kit; Bethesda Laboratories, Montgomery, TX) for lung permeability assessment was quantified using a modified double-ligand method.

TdT-dUTP nick end labeling stain. For detection of apoptotic cells in the lungs of mice infected with Klebsiella, tissue sections were analyzed using the TdT-dUTP nick end labeling (TUNEL) assay. After digestion with 0.5% trypsin, sections were treated with TUNEL reaction mixture using the In Situ Cell Death detection kit (Boehringer, Mannheim, Germany) for 1 h at 37°C in the dark. Slides were then rinsed three times with 1× PBS and incubated with alkaline phosphatase-conjugated fluorescein isothiocyanate-labeled antibody for 30 min at 37°C. Sections were then washed and developed with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluidine.

Caspase-3 activity. Whole lung homogenates were analyzed for active caspase-3 levels using a colorimetric assay following the manufacturer’s instructions (R&D Systems).

Live cell time-lapse imaging. Caspase-3 activity was assessed in live cells using DEVD-NucView 488, which contains a DNA-binding dye coupled to a DEVD peptide that is a specific substrate of caspase-3. Cleavage of the peptide by caspase-3 in cytosol results in nuclear translocation of DNA-binding dye, which emits bright green fluorescence on binding to DNA. Appearance of green fluorescent nuclei of apoptotic cells is monitored and imaged using a fluorescent microscope. Time-lapse images were acquired using IncuCyte, a live cell imaging microscope that fits within the standard carbon dioxide incubator (www.essentialscience.com). Images were captured at 6 h and saved as a JPEG file using software from IncuCyte.

Reconstitution of GM-CSF. Mice were anesthetized, and trachea was exposed; intratracheal recombinant mouse GM-CSF was given at a dose of 10 μg/kg per mouse; and the skin incision was closed with surgical staples.

Statistical analysis. Survival curves were compared using the log-rank test. For other data, statistical significance was determined using two-way ANOVA for Figs. 1–3, 4B, 5, and 6; the rest of the figures were analyzed using one-way ANOVA. All calculations were performed using the Prism 3.0 software program for Windows (GraphPad Software, San Diego, CA).

RESULTS

Bacterial clearance and lung injury and inflammatory cell accumulation in TLR4<sup>−/−</sup> and WT postintratracheal Kp challenge. WT and TLR4<sup>−/−</sup> mice were administered 5 × 10<sup>2</sup> CFU Kp, and then lungs were harvested at 6 and 24 h for determination of Kp CFU. Compared with WT mice, TLR4 mutant mice had significantly higher bacterial burden at both 6 h and especially 24 h postintratracheal Kp (40- and 60-fold, respectively, Fig. 1A). Gram-negative bacterial pneumonia is commonly associated with ALI (11). To quantitate damage to the alveolar epithelium, BAL was performed at 6 and 24 h postintratracheal Klebsiella challenge, and protein leak was quantitated by measuring albumin levels in the BALF. Albumin levels in BALF of WT mice were moderately elevated at 24 h but not 6 h postintratracheal Kp. By comparison, TLR4 mutant mice had significantly higher protein levels in the BALF at both 6 and 24 h, suggesting that TLR4 is important for bacterial clearance as well as maintaining the barrier function of alveolar epithelium.

To further understand the mechanism promoting increased bacterial burden and the significantly higher lung injury observed in the TLR4 mutant mice, we assessed total inflammatory cells in BALF both at 6 and 24 h post-Klebsiella challenge and performed cytopsins and determined the total number of neutrophils at 6 and 24 h. Bacterial challenge resulted in an early increase in total lung leukocytes in WT mice by 6 h that was largely due to an increase in PMN (Fig. 1, C and D). Compared with WT infected animals, TLR4<sup>−/−</sup> mice displayed significantly lower numbers of total cells and lung PMN.
at this time point. By 24 h post-Kp administration, no differences in total leukocytes or PMN were observed between WT and TLR4-lps-d mice.

Lung cell death in TLR4-lps-d and WT mice post-intratracheal Kp challenge. Having observed increased lung injury in TLR4-lps-d mice post-Kp administration, we assessed for evidence of death responses in cells that comprise the alveolar-capillary membrane. In initial studies, we evaluated AEC apoptosis as one mechanism of increased protein leak. WT and TLR4-deficient mice were administered 5 × 10^2 CFU Kp, lungs were harvested at 6 and 24 h, and TUNEL stain was performed. As shown in Fig. 2A, there was evidence of apoptosis of lung cells, including AEC (as indicated by brown staining marked by arrows), in infected WT mice. By comparison, considerably more apoptosis was noted in TLR4 mutant mice 24 h post-Kp challenge. To quantitate the amount of apoptosis, WT and TLR4-deficient mice were administered Kp, lungs were harvested at 6 and 24 h, and caspase-3 activity was measured by calorimetric assay. A modest increase in caspase-3 activity was noted in WT mice at 24 h. Interestingly, lungs from TLR4-lps-d mice had significantly greater caspase-3 activity at both 6 and 24 h post-Kp, indicating that the presence of TLR4

Fig. 1. Bacterial colony-forming units (CFU) and lung injury and inflammatory cell accumulation in wild-type (WT) mice and mice with defective Toll-like receptor (TLR) 4 signaling (TLR4-lps-d) 6 and 24 h post-it Klebsiella pneumoniae (Kp) challenge. Mice were challenged with 5 × 10^2 CFU Kp i.t., and lungs and BAL were harvested at 6 and 24 h. A: lung CFU expressed in mean log10 ± SE. B: protein leak in bronchoalveolar lavage (BAL) measured by albumin enzyme-linked immunosorbent assay (ELISA) at 6 and 24 h post-it Kp. The total no. of inflammatory cells in the BAL was measured using a hemocytometer (C). Hematoxylin and eosin (H & E) stains performed on the cytopsins and the total no. of neutrophils was quantified post-Klebsiella challenge (D). PMN, polymorphonuclear neutrophils. No differences were observed in baseline albumin levels in untreated WT and TLR4-lps-d mice. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with WT mice. Values represent means ± SE from 6–8 animals/group, combined from two separate experiments.

Fig. 2. Lung cell death post-it Kp in WT and TLR4-lps-d mice. TdT-dUTP nick end labeling (TUNEL) staining was performed on lung sections from WT and TLR4-lps-d mice at 24 h post-it Kp. A: representative image from WT and TLR4-lps-d mice treated with 5 × 10^2 CFU Kp, and lungs were harvested at 24 h, and TUNEL stain was performed. As shown in Fig. 2A, there was evidence of apoptosis of lung cells, including AEC (as indicated by brown staining marked by arrows), in infected WT mice. By comparison, considerably more apoptosis was noted in TLR4 mutant mice 24 h post-Kp challenge. To quantitate the amount of apoptosis, WT and TLR4-deficient mice were administered Kp, lungs were harvested at 6 and 24 h, and caspase-3 activity was measured by calorimetric assay. A modest increase in caspase-3 activity was noted in WT mice at 24 h. Interestingly, lungs from TLR4-lps-d mice had significantly greater caspase-3 activity at both 6 and 24 h post-Kp, indicating that the presence of TLR4

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protected against lung cell apoptosis and injury postbacterial challenge.

**GM-CSF expression in lung in vivo and by AEC in vitro post-Kp administration.** GM-CSF is a growth factor with important prosurvival effects on epithelial cells, including AEC (1). To investigate the role of GM-CSF in regulating lung injury and AEC apoptosis, we challenged WT and TLR4 mutant mice with intratracheal Kp and then harvested lungs at 6 and 24 h and measured GM-CSF expression by real-time PCR. Robust expression of GM-CSF mRNA was observed in WT mice post-Kp challenge, whereas a considerably blunted GM-CSF response was noted in Kp-infected TLR4lps-d mice (Fig. 3A). We also examined GM-CSF protein levels in whole lung homogenates at 6 and 24 h postbacterial challenge. An increase in GM-CSF was noted in Kp-challenged WT mice at 6 h, which persisted out to 24 h. By comparison, production of GM-CSF in lung was significantly decreased in TLR4 mutant mice at both 6 and 24 h post-Kp (Fig. 3B). These data indicate that signaling through TLR4 is required for maximal expression of GM-CSF during Kp pneumonia.

AEC are a major cellular source of GM-CSF in the lung (1). Having observed reduced expression of GM-CSF in whole lung in infected TLR4lps-d mice, compared with their WT counterparts, we next examined production of GM-CSF from WT and TLR4 mutant AEC after exposure to heat-killed Kp in vitro. Primary AECs were harvested from WT and TLR4lps-d mice and then incubated with heat-killed Kp at a multiplicity of infection of 10:1. Cellular mRNA and supernatants were harvested after 24 h, and GM-CSF expression was determined by RT-PCR and ELISA. As shown in Fig. 4A, incubation with Kp resulted in a nearly ninefold increase in GM-CSF expression in WT AEC, whereas expression in TLR4lps-d AEC treated with heat-killed Kp was significantly reduced. Differences in Kp-induced GM-CSF protein production between WT and TLR4lps-d AEC was more striking, since the 10-fold increase in GM-CSF observed in WT AEC after Kp exposure was decreased by nearly 90% in TLR4lps-d AEC (Fig. 4B).

**GM-CSF treatment reduces apoptosis of TLR4lps-d AEC apoptosis.** To determine if AEC from TLR4lps-d mice were more susceptible to Kp-induced apoptosis in vitro, and whether GM-CSF reconstitution might protect mutant cells from apoptosis, we assessed real-time caspase-3 activation in primary cells incubated in the presence or absence of GM-CSF. Primary AEC isolated from WT and TLR4 mutant mice were coincubated with heat-killed Kp for 1 h, washed with PBS, and treated with or without GM-CSF. Caspase-3 activity was assessed in live cells at 6 h using the IncuCyte real-time imaging system (www.essenbioscience.com). As shown in Fig. 5,
a nearly twofold greater percentage of TLR4 mutant AEC expressed active caspase-3 at 6 h post-Kp compared with WT cells. Moreover, treatment of these cells with GM-CSF substantially reduced the number of caspase-3-expressing cells compared with that observed in Kp-challenged WT AEC.

Reconstitution of GM-CSF in TLR4-lps-d mice protects against lung injury and bacterial dissemination. To determine if the defect in GM-CSF production observed in TLR4-lps-d mice contributed meaningfully to impaired clearance and lung injury responses observed in these mice during Kp pneumonia, GM-CSF reconstitution experiments were performed. Recombinant murine GM-CSF (R&D Systems) was administered intratracheally (10 ng/kg) concomitant with high-dose Kp (8 × 10^2 CFU) to both WT and TLR4-lps-d mice. Lung, blood, and BAL were harvested at 24 h for assessment of bacterial clearance and lung permeability. As observed previously, significantly greater Kp CFU were observed in the lungs of TLR4-lps-d mice at 24 h post-Kp compared with similarly treated WT mice, and bacteremia was only observed in TLR4 mutant mice but not WT mice (Fig. 6, A and B). Administration of GM-CSF did not alter lung bacterial clearance in WT mice, and resulted in a trend toward improved bacterial clearance in the lungs of TLR4-lps-d mice, although this difference did not achieve statistical significance (Fig. 6A). However, there was a significant reduction in blood CFU (as a measure of systemic dissemination) in TLR4-lps-d mice. Moreover, GM-CSF administration resulted in a substantial reduction in BAL albumin levels in infected TLR4-lps-d mice but not WT mice (Fig. 6C). This suggests that GM-CSF mediates a protective influence on the alveolarcapillary membrane, effects that are disproportionate to changes in bacterial burden within the lung.

To assess the contribution of TLR4 and GM-CSF to apoptotic cell death in vivo, WT and TLR4-lps-d mice were infected with 8 × 10^2 CFU Kp with or without concomitant intratracheal reconstitution with recombinant murine GM-CSF (10 ng/kg). Lungs were harvested at 24 h and fixed in formalin, lung sections were made, and TUNEL staining was performed. As observed previously, WT mice infected with Kp had some evidence of apoptosis, as demonstrated by the presence of brown nuclear staining (Fig. 6D). The majority of cells appeared to be cells with morphology consistent with AEC. The amount of apoptosis in WT mice was unchanged postreconstitution of GM-CSF. In comparison, infected TLR4 mutant mice had significantly more apoptosis, which was substantially decreased after GM-CSF reconstitution. We further quantitated apoptosis by measuring the total number of TUNEL-positive cells per high-power field from each group (Fig. 6E). These findings provide further evidence that GM-CSF prevents apoptosis in the lungs of TLR4-lps-d mice postintratracheal bacterial challenge.

DISCUSSION

ALI and ARDS are commonly seen in patients with severe bacterial pneumonia, and the development of lung injury contributes to increased mortality and morbidity associated with pneumonia. Damage to the alveolarcapillary membrane in bacterial infection can occur as a direct result of pathogen-induced damage or host responses to bacterial components. In this study, we found that TLR4 is required for effective bacterial clearance in Kp pneumonia. We and others have previously shown impaired clearance in TLR4 mutant or TLR4-deficient mice, an effect that is partially attributable to reduced cytokine-mediated lung PMN influx (5, 23). Additionally, TLR4 has been shown to be essential to clear the bacteria.
and prevent lethal lung injury and bacteremia in a murine model of *Pseudomonas pneumonia* (10). However, our study is the first to show the importance of TLR4-dependent GM-CSF signaling in maintaining the integrity of the alveolarcapillary membrane during *Klebsiella* pneumonia. In TLR4 mutant mice, increases in alveolar permeability were temporally associated with greater lung cell death, including AEC, and reduced expression of GM-CSF. The basic mechanism(s) by which TLR4 regulates lung injury responses in pneumonia have not been completely elucidated. One could speculate that TLR4-deficient mice might be protected against lung injury based on reductions in TLR4-driven inflammatory cytokine cascades. Consistent with this notion, we observed reduced neutrophilic recruitment and decreased GM-CSF production in whole lung of TLR4-deficient mice compared with the WT mice postbacterial challenge. However, despite decreased inflammation, we found significantly higher protein leak in TLR4-deficient mice postbacterial challenge compared with the WT animals. Activation of TLR4 has previously been shown to protect epithelium in several noninfectious murine models of lung injury (22, 26). The mechanism of protection in a bleomycin model is believed to be via activation of NF-kB dependent prosurvival pathways in AEC (16). In hyperoxic lung injury, TLR4 is required for activation of protective Nrf-2-dependent antioxidant production (26). In this study, we describe a novel means by which TLR4 contributes to protection against lung injury. Specifically, we found reduced expression of GM-CSF in lungs of TLR4-deficient mice during *Kp* pneumonia, and exogenous administration of recombinant mouse GM-CSF restored lung alveolar barrier function, decreased AEC apoptosis, and limited bacterial dissemination without significantly altering lung clearance responses. This later observation strongly argues against increased alveolar permeability in infected TLR4 mutant mice simply due to increased bacterial burden.

GM-CSF is well recognized for its potent stimulatory effects on hematopoietic cells, including stimulation of both neutrophil and monocyte maturation and release from bone marrow. Compelling experimental evidence supports a key role for GM-CSF in maintaining homeostasis in the lung. For example, GM-CSF promotes alveolar macrophage maturation and antimicrobial function, an effect that is dependent on the transcription factor PU.1 (4). This growth factor

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Fig. 6. Reconstitution of GM-CSF in WT and TLR4-deficient mice. Recombinant mouse GM-CSF (R&D Systems) was administered it (10 μg/kg) concomitant with high-dose *Kp* (8 × 10^2 CFU) to both WT and TLR4-deficient mice. Lungs, blood, and BALF were harvested at 24 h for assessment of lung and blood bacterial clearance (*A* and *B*, respectively), lung permeability as measured by BALF albumin (*C*), and TUNEL staining (*D*). The total no. of TUNEL-positive cells was quantitated from 10 high-power fields (*E*); n = 10–12 mice in each group/experiment, combined from three separate experiments. *P < 0.01 and **P < 0.001. TUNEL staining is representative of three separate mice/group.
also regulates key biological processes in alveolar epithelium. GM-CSF is a mitogen for rodent AEC in primary culture (15, 20). Moreover, GM-CSF exerts cytoprotective effects in several forms on noninfectious lung injury, including bleomycin and hyperoxic lung injury (16, 26), BAL GM-CSF levels inversely correlate with magnitude of lung injury in patients with ALI/ARDS, and administration of GM-CSF in patients with sepsis-induced ALI has been shown to improve PaO₂/FIO₂ ratios (17, 21).

AEC are integral cellular components of innate host defense in the lung and in maintaining the structural and functional integrity of the alveolus. These cells respond to microbial invasion by producing cytokines and chemokines required to amplify innate responses (24). AEC are also the major cellular sources of growth factors, including GM-CSF (20). Our in vitro data suggest that the reduction of GM-CSF in the airspace of TLR4<sup>−/−</sup> mice during Kp infection is partially due to diminished expression of this cytokine by AEC. The autocrine production of GM-CSF appears to contribute to prosurvival responses of these cells, since we observed enhanced caspase-3 activation in Kp-challenged AEC isolated from TLR4 mutant mice in vitro, and GM-CSF administration prevented the activation of caspase-3 in these cells. Consistent with these observations, we found enhanced apoptosis of LPS-treated AEC isolated from GM-CSF<sup>−/−</sup> (C57B/6 background) compared with similarly treated cells from WT mice (Bhan, unpublished observations).

The molecular mechanism by which GM-CSF promotes prosurvival effects in AEC has not been described.

Collectively, our data indicate that TLR4 is a vital component of protective host responses during Gram-negative bacterial pneumonia. Not only is TLR4 signaling required for effective innate antibacterial clearance mechanisms, but it also is necessary for cytoprotection of alveolar epithelium. Clinical trials are ongoing to evaluate the efficacy of GM-CSF administration in established ALI/ARDS or as preventative therapy in patients at risk for the development of ALI.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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