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Interleukin-1 Receptor–Associated Kinase M–Deficient Mice Demonstrate an Improved Host Defense during Gram-negative Pneumonia

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Pneumonia is a common cause of morbidity and mortality and the most frequent source of sepsis. Bacteria that try to invade normally sterile body sites are recognized by innate immune cells through pattern recognition receptors, among which toll-like receptors (TLRs) feature prominently. Interleukin-1 receptor (IL-1R)–associated kinase (IRAK)-M is a proximal inhibitor of TLR signaling expressed by epithelial cells and macrophages in the lung. To determine the role of IRAK-M in host defense against bacterial pneumonia, *IRAK-M*-deficient (*IRAK-M*$^{-/-}$) and normal wild-type (WT) mice were infected intranasally with *Klebsiella pneumoniae*. IRAK-M mRNA was upregulated in lungs of WT mice with *Klebsiella* pneumonia, and the absence of IRAK-M resulted in a strongly improved host defense as reflected by reduced bacterial growth in the lungs, diminished dissemination to distant body sites, less peripheral tissue injury and better survival rates. Although *IRAK-M*$^{-/-}$ alveolar macrophages displayed enhanced responsiveness toward intact *K. pneumoniae* and *Klebsiella* lipopolysaccharide (LPS) *in vitro*, *IRAK-M*$^{-/-}$ mice did not show increased cytokine or chemokine levels in their lungs after infection *in vivo*. The extent of lung inflammation was increased in *IRAK-M*$^{-/-}$ mice shortly after *K. pneumoniae* infection, as determined by semiquantitative scoring of specific components of the inflammatory response in lung tissue slides. These data indicate that IRAK-M impairs host defense during pneumonia caused by a common gram-negative respiratory pathogen.

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INTRODUCTION

Toll-like receptors (TLRs) occupy a central position in the initiation of cellular innate immune responses (1). TLRs can be expressed on the cell surface (TLR1, -2, -4, -5 -6, -10) or in intracellular compartments (TLR3, -7, -8, -9), serving a key role in the early detection of pathogens. Uncontrolled stimulation of TLRs potentially can lead to disproportionate inflammation and tissue injury, such as may occur during sepsis (2). Therefore, TLR signaling is tightly regulated to avoid such detrimental inflammatory responses. Several negative regulators of TLRs have been implicated in preventing excessive TLR signaling, including myeloid differentiation primary-response protein (MyD88), short, ST2, single-immunoglobulin-interleukin (IL)-1 receptor-related-molecule (SIGIRR), toll-interacting protein (TOLLIP), suppressor-of-cytokine signaling (SOCS), A20 and interleukin-1 receptor (IL-1R)–associated kinase (IRAK)-M (3). MyD88 is an adaptor protein essential for signaling via all TLRs, except TLR3 (1,4). In addition, MyD88 mediates intracellular activation after engagement of the type I IL-1 receptor and the IL-18 receptor (5). MyD88 initiates intracellular signaling by recruitment of IRAK-4 and subsequent association and phosphorylation of IRAK-1. IRAK-M inhibits the IRAK-1/IRAK-4 complex and thereby mitigates intracellular responses elicited by all MyD88 dependent receptors (6). Accordingly, *IRAK-M*-deficient (*IRAK-M*$^{-/-}$) macrophages produced higher levels of proinflammatory cytokines upon stimulation with various

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pathogens, TLR ligands or IL-1β (6). Considering its central position in the regulation of TLR and IL-1/IL-18 signaling, IRAK-M likely plays an important role in the host response to bacterial infection. In particular, enhanced IRAK-M expression has been implicated in the immune suppression frequently observed in patients with sepsis, a condition also called LPS tolerance and characterized by a reduced capacity of immune cells to release proinflammatory cytokines upon restimulation. Indeed, *IRAK-M*$^{-/-}$ cells did not become as tolerant to LPS upon reexposure to this bacterial component as WT cells (6), whereas our laboratory recently reported that LPS tolerance observed in healthy humans exposed to intravenous LPS and in patients with gram-negative sepsis correlated with enhanced IRAK-M expression in circulating leukocytes (7,8). Most importantly, in mice with polymicrobial abdominal sepsis, enhanced IRAK-M expression in pulmonary macrophages contributed to a diminished capacity of these cells to respond to *Pseudomonas aeruginosa ex vivo*, which resulted in a strongly impaired host defense response during secondary (following abdominal sepsis) *Pseudomonas* pneumonia (9).

Current knowledge of the functional role of IRAK-M in the host response to invading bacteria and the pathogenesis of sepsis is highly limited and focused primarily on its contribution to LPS tolerance. Pneumonia is the most common cause of sepsis by far (2,10). We argued that IRAK-M could play a pivotal role in host defense against primary bacterial pneumonia, considering its expression in the two most prominent resident cells in the bronchoalveolar space, that is, macrophages and respiratory epithelial cells (6,9,11,12), and its central place in TLR signaling. Therefore, here we induced gram-negative (using *Klebsiella pneumoniae*) pneumonia in *IRAK-M*$^{-/-}$ and WT mice, seeking to establish the contribution of this negative TLR regulator in antibacterial defense in the previously healthy host.

MATERIALS AND METHODS

Mice

Nine- to 11-wk-old C57BL/6 WT mice were purchased from Harlan Sprague Dawley Inc. (Horst, the Netherlands). *IRAK-M*$^{-/-}$ mice, backcrossed > 10 times to a C57BL/6 genetic background were generated as described (6) and bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). Age and gender matched mice were used in all experiments.

Study Design and Sample Harvesting

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pneumonia was induced as described (13,14). Briefly, *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA, USA) was grown for 3 h to mid-logarithmic phase at 37°C using Tryptic Soy broth (Difco, Detroit, MI, USA). Bacteria were harvested by centrifugation at 1,500*g* for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of approximately 10^4 colony-forming units (CFUs)/50 μL, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands) and bacteria were inoculated intranasally. In most experiments, mice were euthanized at predefined time points (n = 7–9 per group at each time point); sample harvesting and processing, and determinations of bacterial loads and cell counts were done as described (13,14). Briefly, mice were anesthetized with Domitor (Pfizer Animal Health Care, Capelle aan der IJssel, the Netherlands; active ingredient: medetomidine) and Nimatek (Eurovet Animal Health, Bladel, the Netherlands; active ingredient: ketamine) and euthanized by heart puncture. Blood was collected in tubes containing heparin. Lungs and spleen were harvested and homogenized in sterile saline (1:5, weight/vol) using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA). CFUs in organ homogenates and blood were determined from serial dilutions plated on blood agar plates, incubated at 37°C for 16 h before colonies were counted. For bronchoalveolar lavage (BAL), the trachea was exposed through a midline incision and BAL fluid (BALF) was harvested by instilling and retrieving 2 × 0.3 mL of sterile phosphate-buffered saline. Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and differential cell counts were performed on cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). For immunoassays in lung homogenates, (unlavaged) lungs were excised, weighed and homogenized in saline (1:5, weight/vol). Lung homogenates were diluted 1:2 in lysis buffer containing 300 mmol/L NaCl, 30 mmol/L Tris, 2 mmol/L MgCl2, 2 mmol/L CaCl2, 2 % Triton X-100 and AEBSF (4-[2-aminoethyl]benzenesulfonyl fluoride), EDTA-Na2, pepstatin and leupeptin (all 8 μg/mL; pH 7.4) and incubated on ice for 30 min and spun down. Homogenates were centrifuged at 1,500*g* at 4°C for 15 min and stored at –20°C until assays were performed. In separate studies, mice were followed for up to 10 d and survival was monitored at least every 12 h (n = 10 per group).

IRAK-M Expression

Lung homogenates were dissolved immediately in RA1 buffer, RNA was isolated as described by the manufacturer (Bioke, Leiden, the Netherlands) and reverse transcribed using oligo dT (Promega, Leiden, the Netherlands) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, the Netherlands). Reverse-transcription-polymerase chain reactions (RT-PCRs) were performed using LightCycler SYBR green I master mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus using the following conditions: 5 min 95°C hotstart, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 5 s, 72°C for

20 s). For quantification, standard curves were constructed by PCR on serial dilutions of a concentrated cDNA, and data were analyzed using LightCycler software. Gene expression is presented as the ratio of the expression of the housekeeping gene β2-microglobulin (B2M). Primers were as follows: B2M; 5′-TGGTC TTTCT GGTGC TTGTC T-3′ and 5′-ATTTT TTTCC CGTTC TTCAG C-3′, IRAK-M; 5′-TGCCA GAAGA ATACA TCAGA CAG-3′ and 5′-TCTAA GAAGG ACAGG CAGGA GT-3′.

Assays

Myeloperoxidase (MPO), tumor necrosis factor (TNF)-α, IL-6, IL-1β, IL-17, IL-22, keratinocyte-derived cytokine (KC/CXCL1), macrophage inflammatory protein 2 (MIP-2/CXCL2), LPS-induced CXC chemokine (LIX/CXCL5), MIP-3α (CCL20) and lipocalin 2 levels were determined by enzyme-linked immunosorbent assay (ELISA) (MPO; Hycult, Uden, the Netherlands; all other: R&D Systems, Abingdon, United Kingdom). Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and lactate dehydrogenase (LDH) were determined with commercially available kits (Sigma-Aldrich, Zwijndrecht, the Netherlands), using a Hitachi analyzer (Roche) according to the manufacturer's instructions.

Pathology

Paraffin lung sections were stained with hematoxylin and eosin as described (15), and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, thrombi, pleuritis and percentage of the lung surface demonstrating confluent (diffuse) inflammatory infiltrate by a pathologist blinded for groups. The total lung inflammation score was expressed as the sum of the scores for each parameter, the maximum being 28.

Stimulation of Primary Alveolar Macrophages

Alveolar macrophages were harvested from *IRAK-M*$^{-/-}$ and WT mice by bronchoalveolar lavage (BAL) (n = 8 per strain) as described (15,16). Cells were resuspended in RPMI 1640 containing 2 mmol/L L-glutamine, penicillin, streptomycin and 10% fetal calf serum in a final concentration of 5 × 10^4 cells/200 μl. Cells were then cultured in 48-well microtiter plates (Greiner, Alphen a/d Rijn, the Netherlands) for 2 h and washed with RPMI 1640 to remove nonadherent cells. Adherent monolayer cells were stimulated with *Klebsiella* LPS (L1519, Sigma, St. Louis, MO, USA; 10 μg/mL), growth arrested (by treatment with mitomycin-C) (Sigma); 50 μg/mL for 60 min at 37°C; 12,5 × 10^6 CFU/mL) intact *K. pneumoniae* (MOI 1:100) (17) or RPMI 1640 for 16 h. Supernatants were collected and stored at –20°C until assayed for cytokines/chemokines. (17,18).

Phagocytosis

Alveolar macrophages were harvested from WT and *IRAK-M*$^{-/-}$ mice (n = 8 per strain) by BAL, washed and resuspended in IMDM containing 2 mmol/L L-glutamine and 10% fetal calf serum to a concentration of 25 × 10^4 cells/mL and cultured overnight. UV-irradiated *K. pneumoniae* were labeled with CFSE and opsonized with 10% normal mouse serum (19). Labeled bacteria were incubated with macrophages for 1 h at 37°C (MOI 100:1) and cells were analyzed using FACSCalibur (Becton Dickinson, Breda, the Netherlands). Phagocytosis index of each sample was calculated: median fluorescence of positive cells × % positive cells.

Statistical Analysis

All values are expressed as mean ± SEM. Comparisons for more than two groups were done with Kruskall-Wallis followed by Dunn multiple comparison tests, and other comparisons with Mann-Whitney *U* tests. Survival was compared by Kaplan-Meier analysis followed by a log-rank test. Analyses were done using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

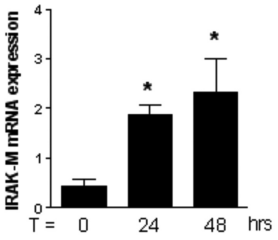

Figure 1. IRAK-M is upregulated during *Klebsiella* pneumonia. IRAK-M mRNA expression was determined in lungs of WT mice before, 24 and 48 h after induction of pneumonia with live 1 × 10^4 CFU *K. pneumoniae*. Gene expression is presented as a ratio of the expression of the housekeeping gene β2 microglobulin. Data are expressed as mean ± SEM; n = 7–8/group; *$P < 0.05$ as compared with t = 0. T (or t), time.

RESULTS

IRAK-M Is Induced during *Klebsiella* Pneumonia

Knowledge of the expression of IRAK-M during primary gram-negative pneumonia is not available. Therefore, we infected WT mice with *K. pneumoniae* via the airways using an established model of severe pneumonia (13,14) and determined pulmonary IRAK-M expression at mRNA and protein level. IRAK-M mRNA was present at low levels in lungs of uninfected mice and displayed strong increases after infection with *K. pneumoniae* (Figure 1).

IRAK-M$^{-/-}$ Mice Demonstrate a Reduced Bacterial Outgrowth and Dissemination during *Klebsiella* Pneumonia

To obtain a first insight into the functional role of IRAK-M in bacterial pneumonia, we harvested lungs, blood, spleen and livers at predefined time points after infection with *K. pneumoniae* for quantitative cultures, seeking to collect data representative for local defense at the primary site of infection and subsequent dissemination and representative for the early (3 and 6 h) and late host response (24 and 48 h, that is, just before the first deaths occurred, see below) (Figures 2A–D). In

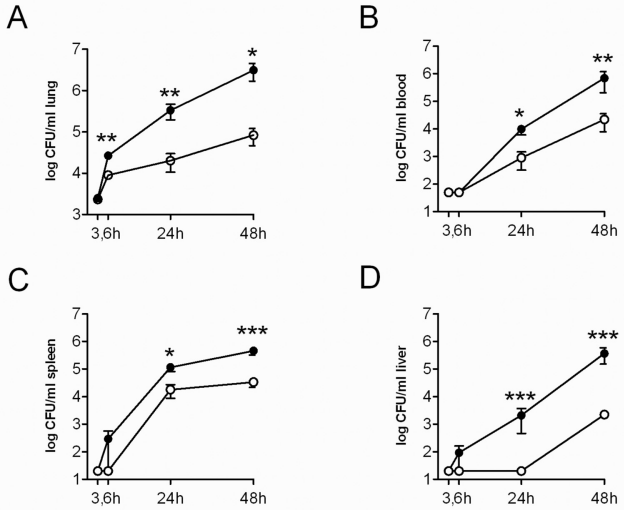

Figure 2. *IRAK-M*$^{-/-}$ mice demonstrate reduced bacterial growth and dissemination during *Klebsiella* pneumonia. WT (closed symbols) and *IRAK-M*-deficient (*IRAK-M*$^{-/-}$) (open symbols) mice were infected intranasally with 1 × 10^4 CFU *K. pneumoniae* and euthanized at 3, 6, 24 and 48 h after induction of pneumonia. Bacterial loads in lung (A), blood (B), spleen (C) and liver (D) were determined. Data are expressed as mean ± SEM; n = 7–9/group; **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 as compared with WT mice.

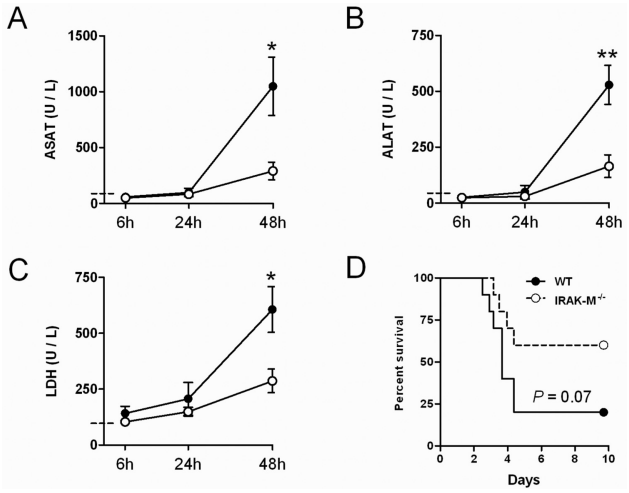

Figure 3. *IRAK-M*$^{-/-}$ mice demonstrate less tissue injury during Klebsiella pneumonia. WT (closed symbols) and *IRAK-M*-deficient (*IRAK-M*$^{-/-}$) (open symbols) mice were infected intranasally with 1 × 10^4 CFU *K. pneumoniae* and euthanized at 6, 24 and 48 h after induction of pneumonia. ASAT (A), ALAT (B) and LDH (C) in plasma were determined. Dotted lines represent mean values of healthy mice (n = 4). Data are expressed as mean ± SEM; n = 7–9/group; **P* < 0.05 and ***P* < 0.01 as compared with WT mice. (D) Survival was observed in WT (closed symbols) and *IRAK-M*$^{-/-}$ (open symbols) mice after intranasal infection with 1 × 10^4 CFU *K. pneumoniae*. n = 10/group. *P* value indicates the difference between groups.

lungs, bacterial loads were similar in *IRAK-M*$^{-/-}$ and WT mice at 3 h after infection. Remarkably, however, at 6 h, *IRAK-M*$^{-/-}$ mice on average had five-fold lower bacterial counts in their lungs than WT mice ($P < 0.01$) and this difference further increased as the infection progressed; at 48 h after infection, bacterial loads were approximately 100-fold lower in *IRAK-M*$^{-/-}$ mice when compared with WT mice ($P < 0.05$). Cultures of blood, spleen and liver remained sterile in all *IRAK-M*$^{-/-}$ mice and all but one of the WT mice during the first 6 h. From 24 h onward, the infection had disseminated from the lungs in all mice, although, clearly, bacterial loads were lower in *IRAK-M*$^{-/-}$ mice in all distant body sites examined. The strongly reduced bacterial burdens in blood, spleen and liver also were reflected in less distant organ damage, that is, the plasma levels of ASAT, ALAT (indicative of hepatocellular injury) and LDH (indicative of cellular injury in general) all were lower in *IRAK-M*$^{-/-}$ mice 48 h after infection (Figures 3A–C). The improved host defense of *IRAK-M*$^{-/-}$ mice also resulted in a survival advantage: 4/10 *IRAK-M*$^{-/-}$ mice died during a 10-d follow-up versus 8/10 WT mice ($P = 0.07$; Figure 3D). Together, these data strongly suggest that IRAK-M impairs antibacterial defense in the lungs upon infection with *Klebsiella*, which subsequently results in enhanced bacterial dissemination and increased organ injury.

IRAK-M$^{-/-}$ Mice Display Increased Lung Inflammation but Unaltered Neutrophil Influx Early after Infection with *Klebsiella* via the Airways

Pneumonia results in local inflammation and inflammatory cell recruitment, which is an integral part of the host immune response (20,21). To obtain insight into the role of IRAK-M herein, we performed semiquantitative analyses of lung histology slides prepared from *IRAK-M*$^{-/-}$ and WT mice 3, 6, 24 and 48 h after infection. These analyses revealed a gradually developing histological picture of pneumonia, as reflected by interstitial inflammation followed by pleuritis and endothelialitis, and, in a later stage, bronchitis and edema. Of interest, *IRAK-M*$^{-/-}$ mice demonstrated significantly more lung inflammation early after infection: both at 3 h ($P < 0.05$) and 6 h ($P < 0.01$), pathology scores were higher in mice lacking IRAK-M relative to WT mice (Figures 4A–F). Especially the almost doubled inflammation score in

IRAK-M$^{-/-}$ mice at 6 h, which, in particular, was caused by the presence of evident bronchitis that, in this phase of the infection, was still absent in all but one WT mice; in addition, *IRAK-M*$^{-/-}$ mice displayed more extensive endothelialitis and edema at early time points, which is remarkable in light of the five-fold lower bacterial load at this time point (which, in theory, provided a less potent proinflammatory stimulus). At later time points after infection, (24 and 48 h) pathology scores had increased considerably in all animals and were no longer any different between mouse strains (Figures 4G–L). Of note, however, at this stage of the infection, *IRAK-M*$^{-/-}$ mice had up to 100-fold fewer bacteria in their lungs, again pointing to an inflammation-enhancing effect of IRAK-M deficiency.

Considering that neutrophils play an important role in innate defense early after *Klebsiella* airway infection (22,23) and in light of the early benefit of *IRAK-M*$^{-/-}$ mice with regard to pulmonary bacterial loads, we determined neutrophil counts in BAL fluid (BALF) harvested from *IRAK-M*$^{-/-}$ and WT mice 3 or 6 h after infection (Figure 4M); in addition, we determined MPO concentrations in whole lung homogenates, providing insight into the total neutrophil content of lung tissue (Figure 4N). These analyses revealed no differences between groups, indicating that the lower pulmonary bacterial loads detected in *IRAK-M*$^{-/-}$ mice 6 h after infection could not be explained by an accelerated recruitment of neutrophils to the primary site of infection. During progressed pneumonia (24 and 48 h after infection), pulmonary MPO concentrations strongly increased in both groups and significantly more so in WT mice (see Figure 4N), probably as a consequence of the much higher bacterial burdens in these animals relative to *IRAK-M*$^{-/-}$ mice.

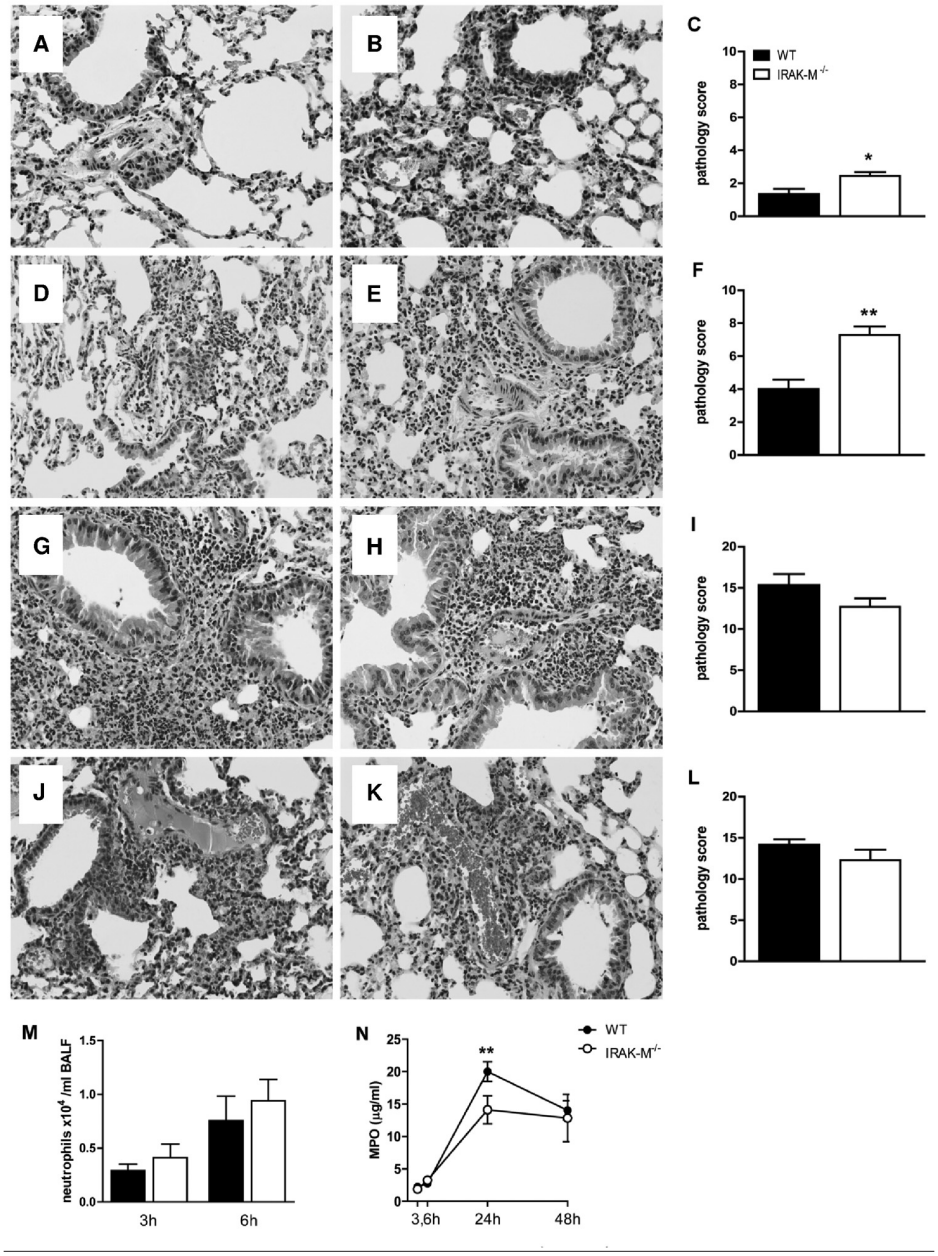

Figure 4. Enhanced lung histopathology in *IRAK-M*$^{-/-}$ mice early after induction of *Klebsiella* pneumonia. Representative lung histology of WT (A, D, G and J) and *IRAK-M*$^{-/-}$ (B, E, H and K) mice at 3 (A–C), 6 (D–F), 24 (G–I) and 48 (J–L) h after intranasal infection with 1 × 10^4 CFU *K. pneumoniae*. The lung sections are representative for 7–9 mice per group per time point. H&E staining, original magnification 20x. Inflammation scores are expressed as mean ± SEM (WT mice: black bars; *IRAK-M*$^{-/-}$ mice: white bars n = 7–9/group). Neutrophil influx into bronchoalveolar lavage fluid (M) and myeloperoxidase (MPO) concentrations in lung homogenates (N) at 3 h to 48 h after infection with 1 × 10^4 CFU of *K. pneumoniae*. Data are expressed as mean ± SEM; n = 7–9/group, **P* < 0.05, ***P* < 0.01 as compared with WT mice.

IRAK-M Deficiency Does Not Influence the Capacity of Alveolar Macrophages to Phagocytsoe *Klebsiella*

Considering the reduced bacterial loads in lungs of *IRAK-M*$^{-/-}$ mice very early after infection, we were interested to examine the impact of IRAK-M deficiency on the capacity of alveolar macrophages to phagocytose *Klebsiella in vitro*. Phagocytosis of *Klebsiella* by *IRAK-M*$^{-/-}$ and WT alveolar macrophages was similar (Figure 5).

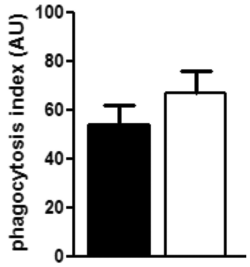

Figure 5. Phagocytosis of *Klebsiella* by WT and *IRAK-M*$^{-/-}$ alveolar macrophages. Phagocytosis of *K. pneumoniae* was determined in alveolar macrophages from WT (closed symbols) and *IRAK-M*$^{-/-}$ (open symbols) mice. Data are expressed as mean ± SEM; n = 8/group, The difference between groups was not significant.

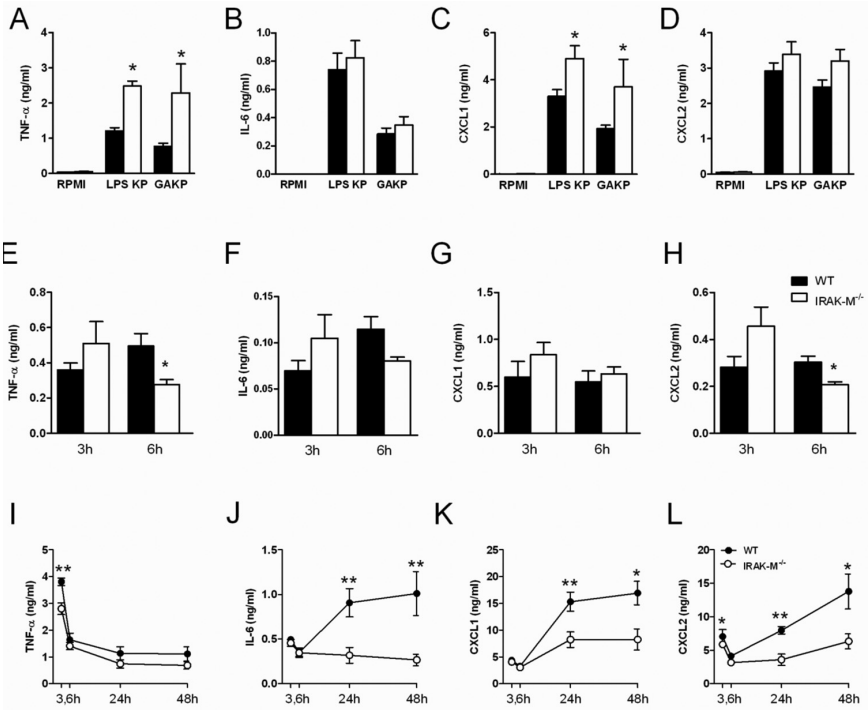

Figure 6. Enhanced TNF-α and CXCL1 release by *IRAK-M*$^{-/-}$ macrophages. TNF-α, IL-6, CXCL1 and CXCL2 concentrations in (A–D) alveolar macrophage supernatants from WT (black bars) and *IRAK-M*$^{-/-}$ (white bars) mice after *ex vivo* incubation with RPMI, LPS from *K. pneumoniae* (LPS KP 10 μg/mL) or growth-arrested *K. pneumoniae* (GAKP MOI 1:100) for 16 h (n = 4/group, **P* < 0.05 as compared with medium stimulation), (E–H) bronchoalveolar lavage fluid and (I–L) lung homogenates from WT (black bars/closed symbols) and *IRAK-M*$^{-/-}$ (white bars/open symbols) mice 3-48 h after infection with 1 × 10^4 CFU of *K. pneumoniae*. Data are expressed as mean ± SEM; n = 7-9/group, **P* < 0.05, ***P* < 0.01 as compared with WT mice.

IRAK-M Deficiency Enhances *Klebsiella*-Induced Cytokine and Chemokine Release by Alveolar Macrophages *Ex Vivo* but Does Not Impact on Mediator Release in Lungs *In Vivo*

IRAK-M$^{-/-}$ bone marrow-derived macrophages have been reported to produce higher levels of proinflammatory cytokines upon incubation with bacteria such as *Escherichia coli*, *Salmonella typhimurium* and *Listeria monocytogenes* (6). To determine the impact of IRAK-M on cytokine and chemokine release by alveolar macrophages exposed to *Klebsiella*, we incubated primary *IRAK-M*$^{-/-}$ and WT alveolar macrophages for 16 h with either growth-arrested *K. pneumoniae* or LPS derived from *K. pneumoniae*, and measured TNF-α, IL-6, CXCL1 and CXCL2 in culture supernatants. *IRAK-M*$^{-/-}$ macrophages released increased amounts of all four mediators, although for IL-6 and CXCL2 the difference with WT cells did not reach statistical significance (Figures 6A–D). Next, we determined whether IRAK-M deficiency influences the release of cytokines and chemokines in the lung during *Klebsiella* pneumonia *in vivo*. First, we focused on early (3 h and 6 h) mediator release into the bronchoalveolar space, considering that alveolar macrophages are the most prominent resident leukocytes there (Figures 6E–H). Although the concentrations of TNF-α, IL-6, CXCL1 and CXCL2 measured in BALF harvested from *IRAK-M*$^{-/-}$ mice 3 h after infection tended to be higher than those detected in BALF from WT mice, the differences between groups were not significant. At 6 h after infection, TNF-α and CXCL2 levels were even lower in BALF obtained from *IRAK-M*$^{-/-}$ mice as compared to WT mice, indicating that the lower bacterial burdens in lungs of the former mouse strain had a greater impact on mediator release than the absence of IRAK-M. To more closely study the contribution of the *Klebsiella* load to pulmonary cytokine and chemokine concentrations, we measured TNF-α, IL-6, CXCL1 and CXCL2 in whole lung homogenates harvested 3 to 48 h after infection, spanning the period of a gradually growing bacterial burden (Figures 6I–L). TNF-α was the only mediator that displayed high concentrations early after infection, decreasing thereafter. In contrast, the lung levels of IL-6, CXCL1 and CXCL2 strongly increased as the infection progressed in WT mice, and, from 24 h onward, the levels of these mediators were all lower in *IRAK-M*$^{-/-}$ mice. Together these data suggest that the bacterial load drives the extent of mediator production in the lungs, overruling the possible inhibiting effect of IRAK-M hereon.

Although IRAK-M expression originally was considered to be restricted to macrophages, recent evidence suggests that IRAK-M likely also is expressed by respiratory epithelial cells (11,12). To examine a possible effect of IRAK-M deficiency on the production of antimicrobial proteins produced by the respiratory epithelium, we measured lipocalin 2 and CCL20, which have been implicated in host defense against respiratory tract infection (24,25).

Early after infection (3 h and 6 h) lipocalin 2 and CCL20 concentrations did not differ in BALF obtained from *IRAK-M*$^{-/-}$ and WT mice (Figures 7A, B). In lung homogenates lipocalin 2 and CCL20 levels were lower in *IRAK-M*$^{-/-}$ mice as the infection proceeded (Figures 7D, E). To obtain further evidence that IRAK-M deficiency did not enhance the responsiveness of respiratory epithelial cells *in vivo*, we measured CXCL5, a CXC chemokine that is produced exclusively by epithelial cells (26,27), in BALF and lung homogenates and found no differences between *IRAK-M*$^{-/-}$ and WT mice (Figures 7C, F). Finally, considering that mucosal immunity in the lung during pneumonia regulated at least in part is by Th17 cytokines (24), we measured IL-17 and IL-22, both of which have been shown to play a protective role in the host response to *Klebsiella* pneumonia (23,28). However, in both mouse strains IL-17 and IL-22, levels were either low or not above baseline concentrations in BALF obtained 3 h or 6 h after infection (data not shown).

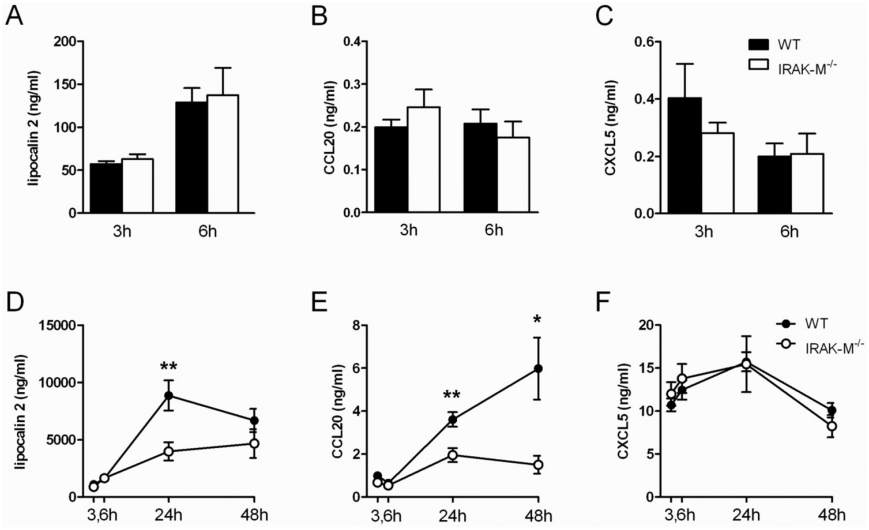

Figure 7. IRAK-M does not affect early epithelial responses. Lipocalin 2, CCL20 and CXCL5 concentrations in (A–C) bronchoalveolar lavage fluid and (D–F) lung homogenates from WT (black bars/closed symbols) and *IRAK-M*$^{-/-}$ (white bars/open symbols) mice 3 h to 48 h after infection with 1 × 10^4 CFU of *K. pneumoniae*. Data are expressed as mean ± SEM; n = 7–9/group, **P* < 0.05 and ***P* < 0.01 as compared with WT mice.

DISCUSSION

Pneumonia represents a persistent and pervasive public health problem. In the United States, respiratory tract infections cause more disease and death than any other infection, and, in the last 50 years, mortality due to pneumonia has not changed significantly (29). Rapid recognition of pathogens that reach the lower respiratory tract is essential for an effective host defense against invading microorganisms (21). IRAK-M inhibits the intracellular IRAK-1 to IRAK-4 signaling complex, and, thereby, all MyD88 dependent receptors (6). A recent study implicated MyD88 as an important mediator of a protective immune response during *Klebsiella* pneumonia (30). Here, we show that IRAK-M strongly impairs host defense during pneumonia caused by *K. pneumoniae*, as reflected by reduced bacterial growth and dissemination and diminished distant organ injury in mice lacking IRAK-M. In accordance, *IRAK-M*$^{-/-}$ mice showed a reduced mortality (40% versus 80% in WT mice), although the difference between groups did not reach statistical significance ($P = 0.07$). Conceivably, larger experimental groups and/or a slightly less severe model using a lower infectious dose would reveal a statistically significant survival advantage for *IRAK-M*$^{-/-}$ mice.

A recent study implicated IRAK-M in the reduced resistance to secondary pneumonia caused by *P. aeruginosa* in mice suffering from polymicrobial peritonitis (9). In this investigation (9), nonlethal septic peritonitis was induced by cecal ligation and puncture to reproduce the immune-compromised state that frequently accompanies sepsis and that is considered to render the host more vulnerable to secondary (nosocomial) infections (2,31,32). Mice subjected to cecal ligation and puncture and subsequently infected with *Pseudomonas* via the airways showed a strongly enhanced bacterial growth and mortality due to the respiratory tract infection when compared with mice that underwent sham surgery. This vulnerable phenotype corresponded with enhanced expression of IRAK-M in lung macrophages harvested from mice with polymicrobial peritonitis and a strongly reduced capacity of these cells to release TNF-α upon incubation with *P. aeruginosa*. In contrast, lung macrophages obtained from *IRAK-M*$^{-/-}$ mice with sepsis released more TNF-α upon exposure to *P. aeruginosa*, which was associated with a markedly improved host defense against this nosocomial pathogen (9). As such, this previous study expanded knowledge on the role of IRAK-M in the immune suppression associated with sepsis (6–8,33). Thus far, the involvement of IRAK-M in the innate immune response to primary bacterial pneumonia in the previously healthy host remained unexplored.

Here, we used an established model of primary pneumonia caused by a common respiratory pathogen in which the bacterial load grows over time at the primary site of infection, followed by dissemination to distant body sites and sepsis, thereby allowing investigation of normal innate defense mechanisms in the respiratory tract in a clinically relevant setting (13,14). Previous studies have documented that the complete absence of MyD88 results in an impaired immune response to *K. pneumoniae* pneumonia (30). Together with the present results, these data suggest that the activity of the MyD88–IRAK-1–IRAK-4 signaling pathway, missing in MyD88$^{-/-}$ mice and less

inhibited in *IRAK-M*$^{-/-}$ mice, is a crucial determinant of antibacterial defense during *Klebsiella* pneumonia. Likely, the influence of IRAK-M on host defense during pneumonia involves its action on multiple MyD88 dependent receptors. Indeed, MyD88 dependent receptors contributing to host defense against *Klebsiella* pneumonia include TLR4 (34,35) and TLR9 (36); the potential roles of other MyD88 dependent TLRs and the IL-18 receptor have not been studied thus far, whereas IL-1 did not play a role of significance (37). Of interest, *IRAK-M*$^{-/-}$ mice demonstrated reduced bacterial loads already very early after infection, suggesting an effect on *Klebsiella* by resident cells. Of note, however, we did not detect a difference in the capacity of *IRAK-M*$^{-/-}$ and WT alveolar macrophages to phagocytose *Klebsiella ex vivo*.

The attenuated growth of bacteria in the lungs was accompanied by an enhanced inflammatory response at the tissue level early after infection (3 h and 6 h), as determined by semiquantitative scores of specific histological alterations characteristic for bacterial pneumonia. Although IRAK-M inhibits cytokine and chemokine production by macrophages stimulated by bacteria or purified TLR ligands *in vitro* (Figure 6) (6,9), IRAK-M deficiency had little if any effect on the pulmonary levels of these mediators during airway infection *in vivo*; during late stages of the infection, cytokine and chemokine levels were even lower in the lungs of *IRAK-M*$^{-/-}$ mice. The strongly reduced bacterial loads in *IRAK-M*$^{-/-}$ mice, providing a diminished proinflammatory stimulus to immune cells in the lung, afford a likely explanation for these lower mediator levels in spite of the absence of the proximal TLR inhibitor IRAK-M. These findings differ from those in *IRAK-M*$^{-/-}$ mice with polymicrobial peritonitis suffering from secondary *Pseudomonas* pneumonia, which showed increased lung levels of several cytokines (9). Apparently, the impact of IRAK-M on cytokine production is more prominent in the already compromised than in the previously healthy host. This may, also, at least in part, explain the fact that IRAK-M deficiency was associated with enhanced neutrophil recruitment to the lungs during secondary pneumonia following peritonitis (9), whereas it did not influence neutrophil influx during primary airway infection (this study). In the current investigation, IRAK-M deficiency did not enhance the production of either lipocalin 2 or CCL20, both antimicrobial proteins produced by the respiratory epithelium implicated in host defense against respiratory tract infection (24,25). In accordance, CXCL5, a chemokine released exclusively by respiratory epithelial cells (26,27), was not altered in *IRAK-M*$^{-/-}$ mice. Of note, *IRAK-M*$^{-/-}$ type II alveolar epithelial cells were reported to release modestly increased amounts of CXCL1 and CXCL2 upon incubation with LPS *in vitro* (38). Nonetheless, our current data suggest that during *Klebsiella* pneumonia *in vivo* IRAK-M deficiency does not affect proinflammatory mediator release by respiratory epithelial cells to a significant extent.

In a very recent investigation, *IRAK-M*$^{-/-}$ mice displayed an exaggerated inflammatory response in their lungs upon infection with influenza A virus (38). In contrast to what we found here, the extent of lung inflammation in *IRAK-M*$^{-/-}$ mice was prolonged over the course of 6 d and associated with an impaired antiviral response and increased lethality (38). Hence, in influenza, pneumonia IRAK-M serves to prevent detrimental inflammation thereby facilitating viral clearance. Clearly, this role is different from the function of IRAK-M during *Klebsiella* pneumonia, where the early inhibition of the lung inflammatory response resulted in enhanced bacterial growth and adversely affected outcome. Previous studies confirm the "double-edged sword" character of innate immunity during lung infection (39,40), wherein not only aberrant activation of leukocytes and epithelial cells, but also of endothelial cells resulting in vascular hyperpermeability, may contribute to collateral damage as a consequence of innate immune activation (41,42).

WT mice purchased from a commercial supplier (and not littermates) were used as controls. As such, differences between *IRAK-M*$^{-/-}$ and WT mice could have been caused in part by differences in composition of the gut microbiome. Additional studies are warranted to examine whether IRAK-M deficiency influences the gut microbiome.

IRAK-M is a negative regulator of TLR signaling that occupies a crucial role in the activation of macrophages upon the first encounter of the immune system with pathogens. Here, we show that IRAK-M becomes upregulated rapidly in the lungs of mice infected with *K. pneumoniae* via the airways. Physiologically positioned to inhibit potentially damaging lung inflammation, IRAK-M dampens the early antibacterial response during *Klebsiella* pneumonia as reflected by lower bacterial counts in *IRAK-M*$^{-/-}$ mice from 6 h after infection onward.

CONCLUSION

Our study is the first to demonstrate a function for IRAK-M in host defense against respiratory tract infection in the previously healthy host. Preliminary experiments performed by our laboratory indicate that IRAK-M also impairs host defense during gram-positive pneumonia (data not shown). Together these data identify IRAK-M as a possible therapeutic target in the treatment of bacterial respiratory tract infection.

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DISCLOSURE

The authors declare they have no competing interests as defined by Molecular Medicine, or other interest that might be perceived to influence the results and discussion reported in this paper.

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