Immunotolerance during bacterial pneumonia and sepsis

Hoogerwerf, J.J.

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Interleukin-1-receptor-associated kinase M deficient mice demonstrate an improved host defense during gram-negative pneumonia

JJ Hoogerwerf, GJW van der Windt, DC Blok, AJ Hoogendijk, AJJ Lammers, AF de Vos, C van ‘t Veer, S Florquin, KS Kobayashi, RA Flavell, T van der Poll

Submitted
Abstract

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) prominently feature. Interleukin-1-receptor-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 intranasally infected with Klebsiella \textit{(K.) pneumoniae}. IRAK-M mRNA and protein were 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. The improved antibacterial defense of IRAK-M−/− mice in vivo was corroborated by an enhanced phagolysosomal fusion capacity of IRAK-M−/− macrophages upon exposure to Klebsiella in vitro. Although IRAK-M−/− alveolar macrophages displayed enhanced responsiveness toward intact \textit{K. pneumoniae} and Klebsiella 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 \textit{K. pneumoniae} infection, as determined by semi-quantitative 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.
Role of IRAK-M in gram-negative pneumonia

Introduction

Toll-like receptors (TLRs) occupy a central position in the initiation of cellular innate immune responses. TLRs can be expressed on the cell surface (TLR1, -2, -4, -5, -6 and -10) or in intracellular compartments (TLR3, -7, -8 and -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. Therefore, TLR signaling is tightly regulated in order 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 IL-1R-associated kinase (IRAK)-M. MyD88 is an adaptor protein essential for signaling via all TLRs, except TLR3. In addition, MyD88 mediates intracellular activation after engagement of the type I IL-1 receptor and the IL-18 receptor. 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. In accordance, IRAK-M deficient (IRAK-M-/-) macrophages produced higher levels of proinflammatory cytokines upon stimulation with various pathogens, TLR ligands or IL-1β. 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 referred to as 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 re-exposure to this bacterial component as wild-type (WT) cells, 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. 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 (P.) aeruginosa ex vivo, which resulted in a strongly impaired host defense response during secondary (i.e. following abdominal sepsis) Pseudomonas pneumonia. 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 primarily focused on its contribution to LPS tolerance. Pneumonia by far is the most common cause of sepsis. 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, i.e. macrophages and respiratory epithelial cells, and its central place in TLR signaling. Therefore, we here induced gram-negative (using *Klebsiella (K.) 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 eleven week 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 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**

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pneumonia was induced by intranasal inoculation of 10^6 colony forming units (CFU) of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Rockville, MD) as described. 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. In separate studies mice were followed for up to 10 days and survival was monitored at least every 12 hours (N=10 per group).

**IRAK-M expression**

Lung homogenates were immediately dissolved 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-minute 95°C hot-start, followed by 40 cycles of amplification (95°C for 15 seconds, 60°C for 5 seconds, 72°C for 20 seconds). 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'-TGGTCTTTCTGGTGCTTGTCT-3' and 5'-ATTTTTTTCCCGTTCTTCAGC-3', IRAK-M; 5'-TGCCAGAAGAATACATCAGACAG-3' and 5'-TCTAAGAAGGACAGGCAGGAGT-3'. Samples for western blotting were boiled at 95 °C for 5 minutes in laemmli buffer and loaded onto SDS-PAGE gels. After electrophoresis the content of the gels was transferred onto Immobilon-PVDF membranes (Millipore, Billerica, MA). The membranes were blocked in 5% Protifar (Nutricia, Zoetermeer, the Netherlands) in TBS-T at room temperature for 60 minutes. Rabbit anti-human IRAK-M (Chemicon, Temecula, CA; cross-reactive with mouse IRAK-M) was diluted 1:500. Anti-Glyceraldehyde-3-PDH (GAPDH; Chemicon) was diluted 1:200. The membranes were incubated overnight at 4°C. Next, the membranes were incubated for 60 minutes with anti-rabbit-HRP conjugated secondary antibody (Cell signalling Technology, Boston, MA) and blots were imaged using Lumilight Plus ECL (Roche, Basel, Switzerland) on a GeneGnome chemiluminescence imager (Syngene, Cambridge, UK).

**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 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), using a Hitachi analyzer (Roche) according to the manufacturer's instructions.

**Pathology**

Paraffin lung sections were stained with hematoxilin and eosin as described, 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. Cells were resuspended in RPMI 1640 containing 2 mM L-glutamine, penicillin, streptomycin and 10% fetal calf serum in a final concentration of 5x10⁶ cells/200 μl. Cells were then cultured in 48-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2 hours and washed with RPMI 1640 to remove non-adherent cells. Adherent monolayer cells were stimulated with Klebsiella LPS (L1519, Sigma, St. Louis, MO;
10 µg/ml), growth arrested (by treatment with mitomycin-C (Sigma); 50 µg/ml for 60 minutes at 37°C; 12.5x10⁶ CFU/ml) intact *K. pneumoniae* (MOI 1:100) or RPMI 1640 for 16 hours. Supernatants were collected and stored at −20°C until assayed for cytokines/chemokines.

**Phagolysosomal fusion**

Peritoneal macrophages were harvested from IRAK-M⁻/⁻ and WT mice as described. *K. pneumoniae* was treated for 60 minutes at 37°C with 50 µg/ml mitomycin-C (Sigma-Aldrich, Zwijndrecht, the Netherlands) to prepare alive but growth-arrested bacteria, opsonised by 30 minutes incubation at 37°C with 10% normal mouse serum and labeled with pHrodo (Invitrogen, Breda, The Netherlands). Labeled bacteria (2.5x10⁸ CFU/ml) were incubated for 1 or 2 hours with macrophages (2.5x10⁵ per well) at 37°C (MOI 1:50) and cells were analyzed using FACSCalibur (Becton Dickinson). Phagolysosomal fusion index of each sample was calculated: mean fluorescence of positive cells x % 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’s 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). Values of P<0.05 were considered statistically significant.

**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 and determined pulmonary IRAK-M expression at mRNA and protein level. IRAK-M mRNA and protein were present at low levels in lungs of uninfected mice and displayed strong increases after infection with *K. pneumoniae* (Figure 5.1A and 5.1B).
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 hours) and late host response (24 and 48 hours, *i.e.* just before the first deaths occurred, see below) (Figure 5.2A-D). In lungs, bacterial loads were similar in IRAK-M-/- and WT mice at 3 hours after infection. Remarkably, however, at 6 hours IRAK-M-/- mice on average had 5-fold lower bacterial counts in their lungs than WT mice (P<0.01) and this difference further increased as the infection progressed; at 48 hours post 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 WT mice during the first 6 hours. From 24 hours 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 were also reflected in less distant organ damage, *i.e.* the plasma levels of ASAT, ALAT (indicative of hepatocellular injury) and LDH (indicative of cellular injury in general) were all lower in IRAK-M-/- mice 48 hours after infection (Figure 5.3A-C). The improved host defense of IRAK-M-/- mice also resulted in a survival advantage: 4/10 IRAK-M-/- mice died during a 10-day follow up versus 8/10 WT mice (P=0.07; Figure 5.3D). 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.

Figure 5.2 IRAK-M<sup>−/−</sup> mice demonstrate reduced bacterial growth and dissemination during *Klebsiella* pneumonia. WT (open symbols) and IRAK-M deficient (IRAK-M<sup>−/−</sup>) (closed symbols) mice were intranasally infected with 1 × 10<sup>4</sup> CFU *K. pneumoniae* and sacrificed at 3, 6, 24 and 48 hours 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 to WT mice.

IRAK-M<sup>−/−</sup> 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<sup>20,21</sup>. To obtain insight into the role of IRAK-M herein, we performed semi-quantitative analyses of lung histology slides prepared from IRAK-M<sup>−/−</sup> and WT mice 3, 6, 24 and 48 hours 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<sup>−/−</sup> mice demonstrated significantly more lung inflammation early after infection: both at 3 (P<0.05) and 6
hours (P<0.01) pathology scores were higher in mice lacking IRAK-M relative to WT mice (Figure 5.4A-F). Especially the almost doubled inflammation score in IRAK-M/- mice at 6 hours, 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, was remarkable in light of the 5-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 hours) pathology scores had increased considerably in all animals and were not different anymore between mouse strains (Figure 5.4G-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.

**Figure 5.3** IRAK-M/- mice demonstrate less tissue injury during Klebsiella pneumonia. WT (open symbols) and IRAK-M deficient (IRAK-M/-) (closed symbols) mice were intranasally infected with 1 x 10^4 CFU K. pneumoniae and sacrificed at 6, 24 and 48 hours 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 to WT mice. (D) Survival was observed in WT (open symbols) and IRAK-M/- (closed symbols) mice after intranasal infection with 1 x 10^4 CFU K. pneumoniae. N=10/group. P-value indicates the difference between groups.
Figure 5.4  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 x 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: open bars; IRAK-M\(^{-/-}\) mice: closed bars n = 7-9/group). Neutrophil influx into bronchoalveolar lavage fluid (M) and myeloperoxidase (MPO) concentrations in lung homogenates (N) at 3-48 hours after infection with 1 x 10\(^4\) CFU of K. pneumoniae. Data are expressed as mean ± SEM; N=7-9/group, * P<0.05, ** P<0.01 as compared to WT mice.
IRAK-M\(^{-/-}\) macrophages demonstrate enhanced phagolysosomal fusion upon incubation with *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 phagolysosomal fusion in macrophages upon exposure to *Klebsiella* in vitro. Interestingly, IRAK-M\(^{-/-}\) macrophages showed increased phagolysosomal fusion as compared with WT macrophages (Figure 5.5).

![Figure 5.5 IRAK-M\(^{-/-}\) macrophages demonstrate enhanced phagolysosomal fusion upon incubation with *Klebsiella*.

Phagolysosomal fusion upon phagocytosis of growth-arrested *K. pneumoniae* was determined in macrophages from WT (open symbols) and IRAK-M\(^{-/-}\) (closed symbols) mice at 37°C (solid lines) and 4°C (dashed lines). Data are expressed as mean ± SEM; N=4-8/group, * P<0.05.

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*. 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 hours 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 (Figure 5.6A-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 and 6 hours) mediator release into the bronchoalveolar space, considering that alveolar macrophages are the most prominent resident leukocytes there (Figure 5.6E-H). Although the concentrations of TNF-α, IL-6, CXCL1 and CXCL2
measured in BALF harvested from IRAK-M<sup>−/−</sup> mice 3 hours after infection tended to be higher than those detected in BALF from WT mice, the differences between groups were not significant. At 6 hours post infection, TNF-α and CXCL2 levels even were lower in BALF obtained from IRAK-M<sup>−/−</sup> mice when 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 – 48 hours post infection, spanning the period of a gradually growing bacterial burden (Figure 5.6L). 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 hours onward the levels of these mediators were all lower in IRAK-M<sup>−/−</sup> 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.

**Figure 5.6** Enhanced TNF-α and CXCL1 release by IRAK-M<sup>−/−</sup> macrophages. 
TNF-α, IL-6, CXCL1 and CXCL2 concentrations in (A-D) alveolar macrophage supernatants from WT (open bars) and IRAK-M<sup>−/−</sup> (closed 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 hours (N=4/group, * P<0.05 as compared to medium stimulation), (E-H) bronchoalveolar lavage fluid and (I-L) lung homogenates from WT (open bars/symbols) and IRAK-M<sup>−/−</sup> (closed bars/symbols) mice 3–48 hours after infection with 1 x 10<sup>3</sup> CFU of *K. pneumoniae*. Data are expressed as mean ± SEM, N=7-9/group, * P<0.05, ** P<0.01 as compared to WT mice.
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. 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. Early after infection (3 and 6 hours) lipocalin 2 and CCL20 concentrations did not differ in BALF obtained from IRAK-M−/− and WT mice (Figure 5.7A and 5.7B). In lung homogenates lipocalin 2 and CCL20 levels were lower in IRAK-M−/− mice as the infection proceeded (Figure 5.7D and 5.7E). 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 exclusively produced by epithelial cells, in BALF and lung homogenates and found no differences between IRAK-M−/− and WT mice (Figure 5.7C and 5.7F). Finally, considering that mucosal immunity in the lung during pneumonia at least in part is regulated by Th17 cytokines, we measured IL-17 and IL-22, which both have been shown to play a protective role in the host response to Klebsiella pneumonia. However, in both mouse strains IL-17 and IL-22 levels were either low or not above baseline concentrations in BALF obtained 3 or 6 hours after infection (data not shown).

Figure 5.7 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 (open bars/symbols) and IRAK-M−/− (closed bars/symbols) mice 3-48 hours after infection with 1 × 10⁶ CFU of K. pneumoniae. Data are expressed as mean ± SEM; N=7-9/group, * P<0.05 and ** P<0.01 as compared to 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. Rapid recognition of pathogens that reach the lower respiratory tract is essential for an effective host defense against invading microorganisms. IRAK-M inhibits the intracellular IRAK-1 – IRAK-4 signaling complex and thereby all MyD88 dependent receptors. A recent study implicated MyD88 as an important mediator of a protective immune response during Klebsiella pneumonia. We here show that IRAK-M strongly impairs host defense during pneumonia caused by K. pneumoniae, as reflected by reduced bacterial growth and dissemination, diminished distant organ injury and an improved survival in mice lacking IRAK-M.

A recent study implicated IRAK-M in the reduced resistance to secondary pneumonia caused by P. aeruginosa in mice suffering from polymicrobial peritonitis. In this investigation, non-lethal septic peritonitis was induced by cecal ligation and puncture in order to reproduce the immune compromised state that frequently accompanies sepsis and that is considered to render the host more vulnerable to secondary (nosocomial) infections. 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. As such, this previous study expanded knowledge on the role of IRAK-M in the immune suppression associated with sepsis. Thus far, the involvement of IRAK-M in the innate immune response to primary bacterial pneumonia in the previously healthy host remained unexplored.

We here 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. Previous studies have documented that the complete absence of MyD88 results in an impaired immune response to K. pneumoniae pneumonia. 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
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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\(^{15,33}\) and TLR9\(^{34}\); 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\(^{35}\). Of interest, IRAK-M\(^{-/-}\) mice demonstrated reduced bacterial loads already very early after infection, suggesting an effect on *Klebsiella* by resident cells. In this respect, our finding of enhanced phagolysosomal fusion in IRAK-M\(^{-/-}\) macrophages upon incubation with *Klebsiella in vitro* provides a possible mechanism that at least in part may explain the improved host defense of IRAK-M\(^{-/-}\) mice in vivo.

The attenuated growth of bacteria in the lungs was accompanied by an enhanced inflammatory response at tissue level early after infection (3 and 6 hours), as determined by semi-quantitative 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 5.6)\(^{6,9}\), 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 even were lower in 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 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 neither enhance the production of 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 exclusively released 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\(^{36}\). 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^{36}. In contrast to what we found here, the extent of lung inflammation in IRAK-M^{−/−} mice was prolonged over the course of 6 days and associated with an impaired antiviral response and increased lethality^{36}. 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.

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. We here show that IRAK-M becomes rapidly upregulated 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 hours after infection onward. Our study is the first to demonstrate a function for IRAK-M in host defense against respiratory tract infection in the previously healthy host.
References