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van der Windt, G.J.W.

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IRAK-M impairs host defense during bacterial pneumonia

Gerritje J.W. van der Windt\textsuperscript{1,2}\*  
Jacobien J. Hoogerwerf\textsuperscript{1,2}\*  
Dana C. Blok\textsuperscript{1,2}  
Arie J. Hoogendijk\textsuperscript{1,2}  
Adriana J.J. Lammers\textsuperscript{1,2}  
Alex F. de Vos\textsuperscript{1,2}  
Cornelis van ‘t Veer\textsuperscript{1,2}  
Sandrine Florquin\textsuperscript{3}  
Richard A. Flavell\textsuperscript{4}  
Tom van der Poll\textsuperscript{1,2}  

\textsuperscript{1}Center for Infection and Immunity Amsterdam (CINIMA), \textsuperscript{2}Center of Experimental and Molecular Medicine, \textsuperscript{3}Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. \textsuperscript{4}Department of Immunobiology, Yale University School of Medicine, New Haven, CT, USA.

\*These authors contributed equally to this article.
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 either Klebsiella (K.) pneumoniae or Streptococcus (S.) 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 and survival rates. Although IRAK-M⁻/⁻ alveolar macrophages displayed enhanced responsiveness toward intact 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 K. pneumoniae infection, as determined by semi-quantitative scoring of specific components of the inflammatory response in lung tissue slides. The phenotype of IRAK-M⁻/⁻ mice during pneumococcal pneumonia was remarkably similar, also demonstrating reduced bacterial growth and dissemination, better survival and enhanced early lung inflammation. These data indicate that IRAK-M impairs host defense during pneumonia caused by two common respiratory pathogens.
Introduction
The innate immune system detects pathogens via a number of pattern-recognition receptors, which recognize conserved motifs that are expressed by micro-organisms (1). Toll-like receptors (TLRs) occupy a central position as pattern recognition receptors in the initiation of cellular innate immune responses (2). 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 (3). 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 (4).

All TLRs, except TLR3, rely on MyD88 for signaling (1, 2); MyD88 also is essential for 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). In accordance, IRAK-M deficient (IRAK-M<sup>−/−</sup>) macrophages produced higher levels of proinflammatory cytokines upon stimulation with various 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 referred to as LPS tolerance and characterized by a reduced capacity of immune cells to release proinflammatory cytokines upon restimulation. Indeed, IRAK-M<sup>−/−</sup> cells did not become as tolerant to LPS upon re-exposure to this bacterial component as wild-type (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 (P.) aeruginosa ex
vivo, which resulted in a strongly impaired host defense response during secondary (i.e. 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 primarily focused on its contribution to LPS tolerance. Pneumonia by far is the most common cause of sepsis (3, 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, *i.e.* macrophages and respiratory epithelial cells (6, 9, 11, 12), and its central place in TLR signaling. Therefore, we here induced gram-negative (*Klebsiella (K.) pneumoniae*) or gram-positive (*Streptococcus (S.) 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 & 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 (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**

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pneumonia was induced by intranasal inoculation of $10^4$ colony forming units (CFU) of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Rockville, MD) or $5 \times 10^4$ CFU of *S. pneumoniae* serotype 3 (ATCC 6303) as described (13-15). 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 (13-15). In separate studies mice were followed for up to 10 days and survival was monitored at least every 12 hours ($n = 10$-$14$ per group).
IRAK-M expression

Lung homogenates were immediately dissolved in RA1 buffer, RNA was isolated as described by the manufacturer (Biogene, 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’-TGAGTCCTTCTGGGTGCTTCT-3’ and 5’-ATTTTTTTCCCCGTTCCTCGC-3’, IRAK-M; 5’-TGCCAGAAGAATACATCAGACAG-3’ and 5’-TCTAAGAA-GGACAGGCGAGGAT-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 (6)) 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), 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-3a (CCL20) and lipocalin 2 levels were determined by ELISA (MPO; Hycult, Uden, the Netherlands; all other: R&D Systems, Abingdon, United Kingdom). Tumor necrosis factor (TNF)-α and IL-6 were measured by cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). 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 (16), 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 (16, 17). Cells were resuspended in RPMI 1640 containing 2 mM L-glutamine, penicillin, streptomycin and 10% fetal calf serum in a final concentration of 5 x 10⁴ 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 non-adherent cells. Adherent monolayer cells were stimulated with Klebsiella LPS (L1519, Sigma, St. Louis, MO; 10 μg/ml), growth arrested (by treatment with mitomycine-C (Sigma); 50 μg/ml for 60 minutes at 37°C; 12,5 x 10⁶ CFU/ml) intact K. pneumoniae (MOI 1:100) (18) or RPMI 1640 for 16 h. Supernatants were collected and stored at -20°C until assayed for cytokines/chemokines.

**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 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 and protein were present at low levels in lungs of uninfected mice and displayed strong increases after infection with *K. pneumoniae* (Figure 1A and B).

**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 1C-F). 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 1G-I). 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 1J). 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 1: IRAK-M is upregulated and impairs the antibacterial response during Klebsiella pneumonia. IRAK-M mRNA (A) and protein (B) expression was determined in lungs of wildtype (WT) mice before, 24 and 48 h after induction of pneumonia with live $1 \times 10^4$ CFU Klebsiella (K.) pneumoniae. Gene expression is presented as a ratio of the expression of the housekeeping gene β2 microglobulin. Protein expression is presented as a ratio of the expression of the housekeeping gene GAPDH. Data are expressed as mean ± SEM; n = 7-8/group; * $P < 0.05$ and ** $P < 0.001$ as compared to t=0. WT (closed symbols) and IRAK-M deficient (IRAK-M$^{-/-}$) (open symbols) mice were intranasally infected with $1 \times 10^4$ CFU K. pneumoniae and sacrificed at 3, 6, 24 and 48 hours after induction of pneumonia. Bacterial loads in lung (C), blood (D), spleen (E) and liver (F), and ASAT (G), ALAT (H) and LDH (I) in plasma were determined; in panels G, H and I the dotted lines represent mean values of healthy mice. 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. (J) Survival was observed in WT (closed symbols) and IRAK-M$^{-/-}$ (open symbols) mice after intranasal infection with $1 \times 10^4$ CFU K. pneumoniae. n = 10/group. P value indicates the difference between groups.

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 (19, 20). To obtain insight into the
role of IRAK-M herein, we performed semi-quantitative analyses of lung histology slides prepared from IRAK-M−/− 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−/− 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 2A-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 2G-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 (21, 22) 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 hours after infection (Figure 2M); in addition, we determined MPO concentrations in whole lung homogenates, providing insight into the total neutrophil content of lung tissue (Figure 2N). These analyses revealed no differences between groups, indicating that the lower pulmonary bacterial loads detected in IRAK-M−/− mice 6 hours after infection could not be explained by an accelerated recruitment of neutrophils to the primary site of infection. During progressed pneumonia (24 and 48 hours after infection), pulmonary MPO concentrations strongly increased in both groups and significantly more so in WT mice (Figure 2N), probably as a consequence of the much higher bacterial burdens in these animals relative to IRAK-M−/− 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
Figure 2: 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 \(1 \times 10^4\) 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 3-48 h after infection with \(1 \times 10^4\) 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.
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 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 3A-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 3E-H). Although the concentrations of TNF-α, IL-6, CXCL1 and CXCL2 measured in BALF harvested from IRAK-M\(^{-/-}\) 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\(^{-/-}\) 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 3I-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 hours 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 (23, 24). Early after infection (3 and 6 hours) lipocalin 2 and CCL20 concentrations did not differ in BALF obtained from IRAK-M\(^{-/-}\)
Figure 3: Enhanced TNF-α and CXCL1 release by IRAK-M\(^{-/-}\) alveolar 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 mg/ml) or growth-arrested \(K.\ pneumoniae\) (GAKP MOI 1:100) for 16 h (\(n = 4\)/group, \(* P < 0.05\) as compared to 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 \(1 \times 10^4\) 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.

and WT mice (Figure 4A and B). In lung homogenates lipocalin 2 and CCL20 levels were lower in IRAK-M\(^{-/-}\) mice as the infection proceeded (Figure 4D and 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 exclusively produced by epithelial cells (25, 26), in BALF and lung homogenates and found no differences between IRAK-M\(^{-/-}\) and WT mice (Figure 4C and F). Finally, considering that mucosal immunity in the lung during pneumonia at least in part is regulated by Th17 cytokines (23), we measured IL-17 and IL-22, which both have been shown to play a protective role in the host response to Klebsiella pneumonia (22, 27). 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).
IRAK-M and bacterial pneumonia

Figure 4: IRAK-M does not affect early epithelial antimicrobial 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 – 48 h after infection with 1 x 10⁴ 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.

IRAK-M similarly impairs host defense during pneumococcal pneumonia

Recently, induction of IRAK-M was reported in lungs of mice with experimentally induced pneumococcal pneumonia (12). This prompted us to examine whether IRAK-M plays a similar detrimental role in the host defense response to respiratory tract infection caused by S. pneumoniae. For this we infected IRAK-M-/- and WT mice with S. pneumoniae via the airways and determined bacterial loads in lungs, blood, spleen and liver at 3, 24 and 48 hours after infection (Figure 5A-D). Similar to what we found in the model of gram-negative pneumonia, IRAK-M-/- mice displayed lower bacterial loads in their lungs (P < 0.01 at both 24 and 48 hours) and a diminished dissemination of the infection to distant organs, together with a delayed mortality (Figure 5E, P = 0.01). The improved antibacterial defense in IRAK-M-/- mice was accompanied by enhanced lung inflammation 3 hours after infection, i.e. at a time point when bacterial loads were still similar (P < 0.01, Figure 6A-C), again resembling the findings in Klebsiella pneumonia. At later time points (24 and 48 hours) pathology scores did not differ anymore between groups (data not shown). At 3 hours after infection lung CXCL1 levels were significantly increased in IRAK-M-/- mice as compared to WT mice (P < 0.01), whereas TNF-α, IL-6, and CXCL2 levels were similar in both groups (Table I). While at 24 hours lung cytokine/chemokine levels were similar, at 48
Figure 5: IRAK-M impairs host response during pneumococcal pneumonia. WT (closed symbols) and IRAK-M\(^{-/-}\) (open symbols) mice were intranasally infected with \(5 \times 10^4\) CFU \textit{S. pneumoniae} and sacrificed at 3, 24 and 48 hours after induction of pneumonia. Bacterial loads in lung (A), blood (B), spleen (C) and liver (D). Data are expressed as mean ± SEM; \(n = 7-8/\text{group}\), * \(P < 0.05\) and ** \(P < 0.01\).

Survival was observed in WT (closed symbols) and IRAK-M\(^{-/-}\) (open symbols) mice after intranasal infection with \(5 \times 10^4\) CFU \textit{S. pneumoniae}. \(n = 13-14/\text{group}\). \(P\) value indicates the difference between groups.
Figure 6: Enhanced lung histopathology in IRAK-M-/- mice early after induction of pneumonoccal pneumonia. Representative lung histology of WT (A) and IRAK-M-/- (B) mice at 3 h after intranasal infection with $5 \times 10^4$ CFU S. pneumoniae. Lung sections are representative for 8 mice per group. H&E staining, original magnification 10x. Inflammation scores (C) are expressed as mean ± SEM (WT mice: black bars; IRAK-M-/- mice: white bars n = 8 mice/group). ** $P < 0.01$ as compared to WT mice.

Table I: Pulmonary cytokine and chemokine concentrations after S. pneumoniae infection.

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<td>IL-6</td>
<td>CXCL1</td>
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<td>3h</td>
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<td>IRAK-M-/-</td>
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<td>4.12 ± 0.50 **</td>
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Data are expressed as mean ± SEM; n = 7-8/group/time point. ** $P < 0.01$, *** $P < 0.001$ vs. WT at the same time point.

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 (28). The consequence of bacterial invasion of the lower respiratory tract depends on the virulence of the organism and the reaction of innate defense mechanisms in the lung (20, 29). IRAK-M is a proximal inhibitor of signaling receptors that rely on MyD88 – IRAK-1 – IRAK-4 for eliciting cellular effects (6). We argued that IRAK-M likely plays a pivotal role in host defense against primary bacterial pneumonia considering its expression in respiratory epithelial cells and macrophages, and
considering that in theory it negatively regulates both the initial response to invading pathogens (by inhibiting MyD88 dependent TLR signaling) and the effector phase of the immune response (by inhibiting signal transduction via the MyD88 dependent IL-1 and IL-18 receptors). We here show that IRAK-M strongly impairs host defense against respiratory tract infection caused by either \textit{K. pneumoniae} or \textit{S. pneumoniae}, as reflected by reduced bacterial growth and dissemination and improved survivals in mice lacking IRAK-M.

Current knowledge of the functional role of IRAK-M in infection is highly limited. Research on IRAK-M has primarily focused on the contribution of this negative regulator to LPS tolerance (6, 30-33), a term indicating the hyporesponsive state of immune cells after pre-exposure to LPS, characterized by a diminished ability to release proinflammatory cytokines upon restimulation by a TLR ligand (34). In patients with sepsis LPS tolerance is a common finding: many studies have documented a reduced cytokine production capacity of circulating leukocytes in this population (8, 35-37). Although the mechanisms that underlie this phenomenon in sepsis are multifactorial and complex (34), enhanced expression of IRAK-M is considered to be involved (6-8, 31). Sepsis-induced leukocyte hyporesponsiveness, also termed “immunoparalysis”, is believed to contribute to the enhanced susceptibility of critically ill patients to secondary nosocomial infections by opportunistic pathogens (3, 38, 39). A recent study implicated IRAK-M in the impaired resistance of the septic host to secondary pneumonia caused by \textit{P. aeruginosa} (9). Specifically, WT mice with non-lethal septic peritonitis induced by cecal ligation and puncture showed a strongly enhanced bacterial growth and mortality due to \textit{P. aeruginosa} pneumonia when compared with mice that underwent sham surgery. This vulnerable phenotype corresponded with enhanced expression of IRAK-M in lung macrophages harvested from WT mice with polymicrobial peritonitis and a strongly reduced capacity of these cells to release TNF-α upon incubation with \textit{P. aeruginosa}. Importantly, lung macrophages obtained from IRAK-M\textsuperscript{-/-} mice with sepsis released more TNF-α upon exposure to \textit{P. aeruginosa}, which was associated with an markedly improved host defense against this nosocomial pathogen in the airways \textit{in vivo} (9). Whereas this elegant study clearly established a role for IRAK-M in secondary pneumonia in the host suffering from abdominal sepsis, the involvement of IRAK-M in the innate immune response to primary pneumonia in the previously healthy host remained unexplored.

We here used two established models of primary pneumonia caused by common respiratory pathogens in which the bacterial load grows over time at the primary
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site of infection, followed by dissemination to distant body sites and sepsis, thereby allowing investigating normal innate defense mechanisms in the respiratory tract in a clinically relevant setting (13-15). Previous studies have documented that the complete absence of MyD88 results in an impaired immune response to both *K. pneumoniae* (40) and *S. pneumoniae* (41). Our current data, showing that elimination of IRAK-M results in improved host defense during *K. pneumoniae* and *S. pneumoniae* induced pneumonia, are in line with these earlier studies (40, 41) and indicate that the activity of the MyD88 – IRAK-1 – IRAK-4 signaling machinery, absent in MyD88−/− mice and enhanced in IRAK-M−/− mice, is a crucial determinant of antibacterial defense during primary bacterial infection of the lungs. Likely, the impact 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 (42, 43) and TLR9 (44); 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 (45). TLRs implicated in a protective immune response against *S. pneumoniae* in the airways include TLR4 (42, 46) and TLR9 (47), whereas TLR1, TLR2 or TLR6 were reported as insignificant (16, 47). In addition, both the type I IL-1 receptor (48) and IL-18 (49) have been found to limit bacterial growth after primary pneumococcal pneumonia. Of note, the fact that the deficiency of individual receptors does not limit bacterial growth during pneumonia does not exclude a role for these receptors in general. Likely, MyD88 is concurrently activated by several receptors upon interaction with a pathogen expressing multiple TLR ligands and consequently IRAK-M deficiency is expected to influence the immune response to *K. pneumoniae* and *S. pneumoniae* by an effect on multiple receptors. In accordance with this notion, in vitro investigations have revealed that TLR2 synergizes with TLR4 and TLR9 for induction of cytokine release by splenocytes stimulated with *S. pneumoniae* (50).

The phenotype of IRAK-M−/− mice was remarkably similar in pneumonia caused by *K. pneumoniae* or *S. pneumoniae*. The attenuated growth of bacteria in the lungs was consistently accompanied by an enhanced inflammatory response at tissue level early after infection (3 and 6 h), 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 3) (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<sup>-/-</sup> mice. The strongly reduced bacterial loads in IRAK-M<sup>-/-</sup> 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<sup>-/-</sup> 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 and CCL20, antimicrobial proteins produced by the respiratory epithelium implicated in host defense against respiratory tract infection (23, 24). In accordance, CXCL5, a chemokine exclusively released by respiratory epithelial cells (25, 26), was not altered in IRAK-M<sup>-/-</sup> mice. Moreover, in preliminary experiments primary respiratory epithelial cells purified from IRAK-M<sup>-/-</sup> mice did not secrete increased quantities of CXCL1 or CXCL2 upon incubation with *K. pneumoniae* (data not shown). Together these data suggest that during pneumonia IRAK-M deficiency does not affect the proinflammatory properties of respiratory epithelial cells to a significant extent.

IRAK-M is a proximal inhibitor of receptors that rely on MyD88 signaling for exerting effects on the cellular interior. We here show that IRAK-M strongly impacts on the outcome of primary gram-negative and gram-positive pneumonia as reflected by attenuated bacterial growth and dissemination and an improved survival in IRAK-M<sup>-/-</sup> mice. Whereas previous studies have identified a clear role for IRAK-M in LPS tolerance and bacterial pneumonia in the immune compromised host, our study is the first to demonstrate a function for IRAK-M in respiratory tract infection in the previously healthy host.

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References


