Cell-specific pattern recognition receptor signaling in antibacterial defense
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Chapter 5

TIR-domain-containing adaptor-inducing interferon-β (TRIF) mediates antibacterial defense during gram-negative pneumonia by inducing Interferon-γ


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Abstract

*Klebsiella pneumoniae* is an important cause of gram-negative pneumonia and sepsis. Mice deficient for TIR-domain-containing adaptor-inducing interferon-β (TRIF) demonstrate enhanced bacterial growth and dissemination during *Klebsiella* pneumonia. We here show that the impaired antibacterial defense of TRIF mutant mice is associated with absent interferon (IFN)-γ production in the lungs. IFN-γ production by splenocytes in response to *K. pneumoniae* in vitro was critically dependent on Toll-like receptor 4 (TLR4), the common TLR adapter myeloid differentiation primary response gene (MyD88) and TRIF. Reconstitution of TRIF mutant mice with recombinant IFN-γ via the airways reduced bacterial loads in lungs and distant body sites to levels measured in wild-type mice, and partially restored pulmonary cytokine levels. The IFN-γ induced improved enhanced antibacterial response in TRIF mutant mice occurred at the expense of increased hepatocellular injury. These data indicate that TRIF mediates antibacterial defense during gram-negative pneumonia at least in part by inducing IFN-γ at the primary site of infection.
Introduction

Globally, pneumonia is a common cause of morbidity and mortality and the most common cause of sepsis (1-3). The emerging antibiotic resistance among gram-negative pathogens, including Enterobacteriaceae such as Klebsiella (K.) pneumoniae, is an issue of major concern, since therapeutic options are limited and infections with these pathogens are associated with an unfavorable outcome (3, 4). K. pneumoniae is a common sepsis pathogen in humans, in particular in the context of lower respiratory tract infection (2).

Pathogens entering the lower airways are detected by innate immune cells via pattern recognition receptors, among which the family of Toll-like receptors (TLRs) features prominently; this interaction initiates the early immune response (5). TLR signaling can proceed via two different routes that are dependent on myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF) respectively (6). MyD88 is the universal adaptor for all TLRs except TLR3 and leads to NF-κB and MAP kinase activation and the induction of inflammatory cytokines. TRIF is the sole adaptor for TLR3 and in addition contributes to TLR4 signaling, leading to the activation of NF-κB and Interferon regulatory factor 3 (IRF3) and the induction of type I interferon (IFN) and inflammatory cytokine production (6). Notably, TLR4, that recognizes lipopolysaccharide (LPS), first activates the MyD88-dependent pathway before it initiates downstream signaling via the TRIF-dependent pathway once TLR4 complex is transported to the endosome for degradation (7). However, activation of both pathways is necessary for the induction of inflammatory cytokines via TLR4 (7).

We previously reported about the crucial role of the TLR adaptors MyD88 and TRIF during K. pneumoniae infection and their differential contribution to the host response in different body compartments (8, 9). In these studies we noted that mice deficient for TRIF were incapable of IFN-γ production at the primary site of infection (unpublished data). IFN-γ is an important cytokine for innate and adaptive immunity that influences a wide array of immunologically relevant cellular programs, such as the enhancement of leukocyte attraction, up-regulation of pathogen recognition, antigen processing and presentation, and microbicidal effector cell functions (10). A previous report demonstrated the importance of IFN-γ for antibacterial defense and survival during K. pneumoniae by the use of IFN-γ gene deficient mice (11, 12). Moreover, IFN-γ deficient mice were more susceptible to airway infection with Legionella pneumophila and Burkholderia pseudomallei (13, 14), and therapeutic administration of recombinant IFN-γ was beneficial in several models of experimental respiratory tract infection (15, 16). Also, rIFN-γ demonstrated a beneficial effect in several human studies when used as an adjunctive therapy for opportunistic pathogens (17-21).

We here report the impact of TRIF deficiency on pulmonary IFN-γ production during Klebsiella pneumonia. Furthermore, we explored to which extent the absence of local IFN-γ production during K. pneumoniae pneumonia in TRIF deficient mice contributes to their susceptible phenotype. We demonstrate that TRIF dependent signaling is crucial for IFN-γ production in vivo and in vitro and that reconstitution
of IFN-γ levels in the airways improves antibacterial defense in TRIF deficient but not in wild-type (WT) mice.

**Materials and methods**

**Animals**
TRIF mutant mice, generated on a C57Bl/6 genetic background (22), were provided by Dr B. Beutler (Center for the Genetics of Host Defense, University of Texas Southwestern Medical Center, Texas). MyD88 deficient (Myd88−/−) (23) and Tlr4−/− mice (24) were provided by Dr. S. Akira (Research Institute for Microbial Diseases, Osaka, Japan) and backcrossed > 8 times to a C57Bl/6 genetic background. All gene deficient mice were bred in the animal facility of the Academic Medical Center (Amsterdam, the Netherlands). Age- and sex matched WT C57Bl/6 control mice were obtained from Harlan Nederland (Horst, the Netherlands). Mice were infected at 10-12 weeks of age. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

**Induction of pneumonia and sampling of organs**
Pneumonia was induced by intranasal inoculation with ~1 x 10^4 colony forming units (CFU) of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA) (8,9). Mice were sacrificed at the indicated time points after infection and organs were harvested and processed exactly as described (8, 25). In the reconstitution experiment, mice were administered 50 ng of recombinant mouse IFN-γ (rIFN-γ) (R&D systems, Abington, United Kingdom) or vehicle (0.1% human serum albumin in sterile saline) intranasally 30 minutes before and 24 hours after inoculation; mice were euthanized after 48 hours of infection.

**Quantitative RT-PCR**
RNA was isolated from lung homogenates using the Nucleospin RNA II kit (Machery-Nagel, Duren, Germany). Total RNA was reverse transcribed using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, The Netherlands). Quantitative PCR of *Ifng* gene product was performed as described (26). Data was analyzed using the LinRegPCR program. Results were normalized to β2m transcript.

**In vitro studies**
Splenocytes were obtained, seeded at a density of 500,000 cells per well and cultured exactly as described (27). Cells were stimulated for 48 hours in at least quadruplicate with the indicated concentrations of mitomycin C-treated (0.05 mg/ml) (Sigma-Aldrich) growth-arrested *K. pneumoniae* diluted in RPMI medium without antibiotics, LPS derived from *Klebsiella pneumoniae* (Sigma) (100 ng/ml) or ultrapure *Escherichia coli* O111 B4 LPS (Invivogen) (100 ng/ml) diluted in RPMI medium with antibiotics in a final volume of 200 microliter. Supernatants were stored and analyzed for cytokine concentrations by ELISA.
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Assays
IFN-γ levels in cell supernatants and lung levels of IL-1β, CXCL1, CXCL2 and CCL2 were measured by ELISA (R&D Systems, Minneapolis, MN and Invitrogen, Breda, the Netherlands). Lung levels of IFN-γ, TNF-α, IL-6 and IL-10 were measured by using a cytometric bead array multiplex assay (BD Biosciences). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using kits from Sigma and a Hittachi analyzer (Boehringer Mannheim).

Histopathology
Histologic examination of lungs and liver was performed exactly as described (25, 28). Granulocyte immunohistochemic stainings were prepared using a FITC-labeled anti-mouse Ly6-C/G mAb (BD Biosciences, San Jose, CA) exactly as described before (27).

Statistical analysis
Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation (in vivo experiments) or as means ± standard error of the mean (tables, cell stimulation experiments); Bacterial loads are expressed as scatter plots, each symbol representing an individual mouse, with horizontal lines indicating medians. For experiments with 2 groups, the Mann–Whitney U test was used to determine statistical significance. For experiments with > 2 groups, the Kruskall-Wallis test was used, followed by Mann–Whitney U tests to compare individual genetically modified groups with the WT or TRIF mutant control group when appropriate. Fisher’s exact test was used to determine if the proportion of positive test results was different. These analyses were done using GraphPad Prism (San Diego, CA). p < 0.05 was considered statistically significant.

Results

IFN-γ production is impaired in TRIF mutant mice during Klebsiella pneumonia

In our previous studies on the role of TRIF during K. pneumoniae airway infection we demonstrated that TRIF mutant mice have an impaired antibacterial defense as illustrated by significantly higher bacterial loads in lungs, blood and spleen (8); in these investigations we also observed higher bacterial loads in livers of TRIF mutant mice and TRIF bone marrow chimeras lacking TRIF in hematopoietic cells (supplementary Figure 1A,B). We noticed in a multiplex cytokine assay performed on whole lung homogenates that IFN-γ levels remained undetectable in TRIF mutant mice throughout the infection (<5 pg/ml), while in WT mice lung IFN-γ concentrations increased after Klebsiella inoculation, peaking after 24 hours (p < 0.05 to 0.001 for the difference between groups, figure 1A). TRIF mutant mice also showed strongly reduced IFN-γ mRNA expression in lungs during Klebsiella pneumonia (p < 0.01 versus WT mice, figure 1B).
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Figure 1: TRIF mediates IFN-γ production during *K. pneumoniae* airway infection. WT and TRIF mutant mice (n=7-8 per group) were infected with ~1 x 10^4 CFU *K. pneumoniae* and sacrificed at designated time points. IFN-γ levels in lungs of mice were determined by cytometric bead assay (A) and quantitative real-time RT-PCR (qRT-PCR) (B). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation (A,B) or as mean (SE) (C). *p* < 0.05, **p** < 0.01 determined with Mann–Whitney U test. ### *p* < 0.001 determined with Fisher’s exact test.

*IFN-γ* production in response to Klebsiella is TLR4 dependent via both Myd88 and TRIF

Next, we stimulated splenocytes, as a source of IFN-γ producing cells, with growth-arrested *K. pneumoniae* in vitro. In a pilot-study, we observed significantly impaired IFN-γ secretion by TRIF mutant cells stimulated with either 2 x 10^5 or 2 x 10^6 bacteria (data not shown). We repeated this experiment, this time including splenocytes of *Tlr4/-* and *Myd88/-* mice in addition to splenocytes from TRIF mutant and WT mice. IFN-γ production in response to growth-arrested *K. pneumoniae* was most severely impaired in *Myd88/-* cells, followed by *Tlr4/-* and then TRIF mutant cells (figure 2A, *p* < 0.05 to 0.01 compared to WT cells). In addition, we stimulated cells with LPS derived from *K. pneumoniae* or ultra-purified LPS derived from *E. coli*, and found virtually absent IFN-γ release by *Tlr4/-*, *Myd88/-* and TRIF mutant cells (figure 2B, *p* < 0.01 versus WT cells).

*Antibacterial defense of TRIF mutant mice can be restored by local treatment with IFN-γ*

To test if the strongly reduced pulmonary IFN-γ levels contribute functionally to the impaired antibacterial defense of TRIF mutant mice we treated WT and TRIF mutant mice with IFN-γ intranasally 30 minutes before and 24 hours after infection with *Klebsiella*; we used 48 hours of infection as pre-defined endpoint since this was the time point at which the enhanced growth of *Klebsiella* in TRIF mutant relative to WT mice was most clear (8). While TRIF mutant mice treated with vehicle displayed undetectable pulmonary IFN-γ concentrations, confirming the results presented in figure 1A, TRIF mutant mice administered with rIFN-γ had lung IFN-γ levels that were similar to those measured in WT mice (figure 3A); WT mice that received rIFN-γ had significantly higher lung levels than WT mice treated with vehicle (*p* <
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Figure 2: IFN-γ secretion by splenocytes is dependent on TLR4, MyD88 and TRIF. Splenocytes derived from WT, Tlr4−/−, Myd88−/− and TRIF mutant mice were stimulated with different concentrations of growth arrested K. pneumoniae, and LPS derived from E. coli or K. pneumoniae (n=4-6 for each condition), and IFN-γ levels were determined after 48 hours. Data are expressed as mean (SE). * p < 0.05, ** p < 0.01 determined with Mann–Whitney U test (performed as post hoc following Kruskal-Wallis test).
0.05). We reproduced the previously described phenotype in TRIF mutant mice (8), showing 100-1000 fold higher bacterial loads in their lungs relative to WT mice, together with increased bacterial dissemination to blood and spleen (figure 3B-D, p < 0.001). Importantly, we observed a spectacular improvement of antibacterial defense in rIFN-γ treated TRIF mutant mice compared to vehicle treated TRIF mutant mice (p < 0.01 to 0.001), as reflected by bacterial loads similar to WT mice in all organs. Of note, we observed no effect on bacterial burdens in WT mice treated with rIFN-γ compared to vehicle treated WT mice (figure 3B-D).

Figure 3: Administration of rIFN-γ via the airways restores antibacterial defense in TRIF mutant mice. WT and TRIF mutant mice were infected with ~1 x 10^4 CFU K. pneumonia; 50 ng recombinant IFN-γ or vehicle was administered intranasally 30 minutes before infection and 24 hours thereafter (n=8 mice each group). Mice were sacrificed after 48 hours of infection. IFN-γ levels in lung homogenates 48 hours after infection (A) depicted as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. Bacterial loads in lung (B), blood (C) and spleen (D) 48 hours after infection. Each symbol represents an individual mouse, horizontal lines represent medians. ** p < 0.01, *** p < 0.001 vs WT mice treated with vehicle, ## p < 0.01, ### p < 0.001 vs TRIF mutant mice treated with vehicle determined with Mann-Whitney U test, Fisher’s exact test was used in panel 1A for comparison between TRIF mutant groups (performed as post hoc after following Kruskal-Wallis test).
Impact of IFN-γ treatment on the inflammatory response to pneumonia

To obtain insight in the extent of local inflammation at the primary site of infection in TRIF mutant and WT mice, and the effect of rIFN-γ treatment hereon, we semi-quantitatively scored lung histopathology of tissue samples harvested 48 hours after infection, focusing on key histological features characteristic for severe pneumonia. While total lung histopathology scores were not different between groups (table 1), rIFN-γ treated TRIF mutant and WT mice had more signs of bronchitis and less signs of pleuritis when compared to their respective vehicle treated controls (table 1, \( p < 0.05 \) compared to the respective controls and figure 4A-F). Neutrophil recruitment to the lungs, measured as the percentage of Ly-6+ positive lung cell surface, was significantly higher in vehicle treated TRIF mutant mice compared to vehicle treated WT mice at this late stage of infection (table 1, \( p < 0.01 \)). Administration of rIFN-γ reduced total neutrophil numbers in lung tissue of TRIF mutant mice similar to those measured in WT mice (\( p < 0.05 \) compared to vehicle treated TRIF mutant mice); rIFN-γ treatment did not influence lung neutrophil counts in WT mice (table 1). Moreover, when the Ly-6 stainings were studied in detail, the number of intrabronchial neutrophils appeared to be larger after rIFN-γ treatment (Figure 4E-H). We next determined the effect of rIFN-γ treatment on the induction of proinflammatory cytokines (TNF-α, IL-1β, IL-6), the anti-inflammatory cytokine IL-10 and chemokines CXCL1, CXCL2 and CCL2 in whole lung homogenates. TRIF mutant mice demonstrated reduced levels of TNF-α, IL-1β, CXCL1, CXCL2 and CCL2 relative to WT mice (table 2, \( p < 0.05 \) to 0.001). Treatment of TRIF mutant mice with rIFN-γ partially restored the inflammatory profile with the exception of IL-1β: TNF-α and CXCL2 were not significantly different from vehicle treated WT mice, levels of CXCL1 and CCL2 were still significantly lower although differences were smaller (\( p < 0.05 \) to 0.01 compared to vehicle treated WT mice). The change in levels of inflammatory cytokines and chemokines after treatment with rIFN-γ of TRIF mutant mice was significant for TNF-α, IL-6, CXCL2 and CCL2 compared to vehicle treated TRIF mutant mice (table 2, \( p < 0.05 \) to 0.001 between groups).

IFN-γ deficiency protects TRIF mutant mice from liver injury

*Klebsiella* induced pneumonia derived sepsis is associated with hepatocellular injury, as reflected by increased plasma concentrations of AST and ALT (8, 29). TRIF mutant mice had lower AST and ALT plasma levels 48 hours after infection when compared with WT mice (\( p < 0.01 \), figure 5A,B) as well as fewer signs of liver inflammation as determined by liver histopathology scores (\( p < 0.01 \), figure 5C, Supplemental figure 2). Remarkably, rIFN-γ treatment significantly increased AST and ALT levels in TRIF mutant mice compared to vehicle treated TRIF mutant mice (\( p < 0.01 \) to 0.001) to levels similar to those measured in WT mice. In WT mice, rIFN-γ treatment reduced transaminase levels, significantly so for AST (\( p < 0.05 \), figure 5A).
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Table 1: Histological scores

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>WT</th>
<th>TRIF mutant</th>
<th>TRIF mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle</td>
<td>rIFN-γ</td>
<td>vehicle</td>
<td>rIFN-γ</td>
</tr>
<tr>
<td>Total pathology score lung</td>
<td>14.5 (0.6)</td>
<td>13.8 (0.8)</td>
<td>14.5 (1.2)</td>
<td>13.1 (0.6)</td>
</tr>
<tr>
<td>Pneumonia % of lung surface</td>
<td>15 (3)</td>
<td>6 (4)</td>
<td>22 (6)</td>
<td>7 (3)</td>
</tr>
<tr>
<td>Interstitial inflammation</td>
<td>3.1 (0.1)</td>
<td>3.0 (0.5)</td>
<td>2.8 (0.7)</td>
<td>2.4 (0.3)</td>
</tr>
<tr>
<td>Oedema</td>
<td>2.8 (0.2)</td>
<td>2.5 (0.3)</td>
<td>3.4 (0.5)</td>
<td>3.0 (0.2)</td>
</tr>
<tr>
<td>Endothelialitis</td>
<td>2.5 (0.2)</td>
<td>2.9 (0.1)</td>
<td>3 (0.2)</td>
<td>2.6 (0.2)</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>2.9 (0.1)</td>
<td>3.5 (0.2)*</td>
<td>2.6 (0.3)</td>
<td>3.8 (0.2)#</td>
</tr>
<tr>
<td>Pleuritis</td>
<td>1.8 (0.3)</td>
<td>1.3 (0.2)*</td>
<td>1.5 (0.3)</td>
<td>0.8 (0.2)#</td>
</tr>
<tr>
<td>Ly6+ % of total lung surface</td>
<td>2.3 (0.4)</td>
<td>2.1 (0.5)</td>
<td>8.6 (1.2)**</td>
<td>3.9 (0.8)#</td>
</tr>
</tbody>
</table>

WT and TRIF mutant mice were infected with 1x10^4 CFU K. pneumoniae and 50 ng recombinant IFNγ was administered intranasally upon infection and after 48 hours. Histological scores determined 48 hours after infection. Total pathology score is the sum of the histological subscores determined as described in the methods. Data are mean (SE) of 7–8 mice per group. *p < 0.05, **p < 0.01 compared to vehicle treated WT mice. #p < 0.05 rIFN-γ treated TRIF mutant mice compared to vehicle treated TRIF mutant mice.

Table 2: Inflammatory response

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>WT</th>
<th>TRIF mutant</th>
<th>TRIF mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle</td>
<td>rIFN-γ</td>
<td>vehicle</td>
<td>rIFN-γ</td>
</tr>
<tr>
<td>TNF-α</td>
<td>892 (245)</td>
<td>760 (57)</td>
<td>191 (48)**</td>
<td>501 (115)#</td>
</tr>
<tr>
<td>IL-1β</td>
<td>7434 (642)</td>
<td>4950 (753)*</td>
<td>4168 (731)**</td>
<td>4525 (557)**</td>
</tr>
<tr>
<td>IL-6</td>
<td>1914 (451)</td>
<td>2030 (624)</td>
<td>2446 (398)</td>
<td>1507 (216)#</td>
</tr>
<tr>
<td>IL-10</td>
<td>14 (2)</td>
<td>11 (1)</td>
<td>14 (2)</td>
<td>bd</td>
</tr>
<tr>
<td>CXCL1</td>
<td>12586 (1899)</td>
<td>9453 (1645)</td>
<td>3625 (871)**</td>
<td>4255 (828)*</td>
</tr>
<tr>
<td>CXCL2</td>
<td>20553 (6546)</td>
<td>38048 (7157)</td>
<td>6432 (1532)*</td>
<td>28943 (5785)###</td>
</tr>
<tr>
<td>CCL2</td>
<td>4619 (541)</td>
<td>3718 (366)</td>
<td>1841 (210)***</td>
<td>2126 (240)**#</td>
</tr>
</tbody>
</table>

WT and TRIF mutant mice were infected with 1x10^4 CFU K. pneumoniae and 50 ng recombinant IFNγ was administered intranasally upon infection and after 48 hours. Homogenates were prepared from right lungs. Cytokine and chemokine levels are presented in pg/ml lung homogenate. Data are mean (SE) of 7–8 mice per group. Bd= below detection.*p < 0.05, **p < 0.01, ***p < 0.001 compared to vehicle treated WT mice. #p < 0.05. ##p < 0.01, ###p < 0.001 rIFN-γ treated TRIF mutant mice compared to vehicle treated TRIF mutant mice.
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Figure 4: Effect of IFN-γ treatment on lung pathology. WT and TRIF mutant mice were infected with ~ 1x 10⁴ CFU K. pneumonia; 50 ng recombinant IFN-γ or vehicle was administered intranasally 30 minutes before infection and 24 hours thereafter. Mice were sacrificed after 48 hours of infection. Representative lung histology (H&E staining) of WT mice treated with vehicle (A), WT mice treated with rIFN-γ (B), TRIF mutant mice treated with vehicle (C) and TRIF mutant mice treated with rIFN-γ (D). In each upper panel arrows indicate signs of bronchitis (original magnification 10x) and in the lower panel asterisks indicate pleuritis (original magnification 20x). Representative lung histology (Ly-6 staining, indicating neutrophils) of lungs of WT mice treated with vehicle (E), WT mice treated with rIFN-γ (F), TRIF mutant mice treated with vehicle (G) and TRIF mutant mice treated with rIFN-γ (H), original magnification 10x.
Figure 5: TRIF mutant mice have attenuated liver injury that increases after rIFN-γ treatment. WT and TRIF mutant mice were infected with ~1 x 10^4 CFU *K. pneumonia*; 50 ng rIFN-γ or vehicle was administered intranasally 30 minutes before infection and 24 hours thereafter. Mice were sacrificed after 48 hours of infection. AST (A) and ALT (B) plasma levels and liver histopathology scored as described in the methods (C) expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. *p < 0.05, **p < 0.01, ***p < 0.001 determined with Mann-Whitney U (performed as post hoc following Kruskal-Wallis test).

Discussion

*K. pneumoniae* is a clinically important gram-negative bacterium in pneumonia and one of the pathogens that causes major concern because of increasing antimicrobial resistance rates, limiting therapeutic options (2-4, 30). Previous research has documented the importance of TLR signaling for host defense during *K. pneumoniae* pneumonia, notably of TLR4, TLR2 and TLR9 (25, 31, 32), and we and others previously described the pivotal role for the TLR-adapters MyD88 and TRIF herein (8, 33). Given our discovery that in the absence of TRIF lung levels of IFN-γ were undetectable during the course of *K. pneumoniae* airway infection we here explored the functional importance thereof. Our main findings were that indeed TRIF is crucial for IFN-γ production in response to *K. pneumonia*, together with TLR4 and MyD88, and that reconstitution of TRIF mutant mice with rIFN-γ improves antibacterial defense to the level of WT mice, but at the expense of enhanced liver injury.

Earlier, we and others described the susceptible phenotype of TRIF deficient mice in *Klebsiella* pneumonia, marked by a clearly impaired antibacterial defense with a 100-1000 fold increase in bacterial loads 48 hours after infection, a finding that we reproduced in the current report (8, 25, 33). The early inflammatory response of mice partially or fully deficient for TRIF is characterized by impaired neutrophil
influx probably as a result of impaired CXCL1 secretion and lower levels of TNF-α and IL-6. However, during the course of the infection and in response to higher bacterial loads all of these cytokines gradually increased in spite of (partial) TRIF deficiency (8). Notably, in the current study CXCL1, CXCL2 and TNF-α levels were still reduced in TRIF mutant mice 48 hours post infection, while lung neutrophil numbers as determined by immunohistochemistry were significantly higher. This is probably due to the very high bacterial numbers present in TRIF mutant mice at this moment, leading to tissue injury and neutrophil attraction via mechanisms other than provided by the chemoattractant gradient by the afore mentioned mediators. Remarkably, however, IFN-γ levels remained virtually undetectable in TRIF mutant mice throughout, which formed the rationale for the current study. We hypothesized that deficient IFN-γ production could at least in part be responsible for the impaired antibacterial defense of TRIF mutant mice, considering that IFN-γ is a powerful pleiotropic cytokine that during bacterial infection can enhance leukocyte attraction, pathogen recognition, antigen processing and presentation, and microbicidal effector cell functions (10). We extended our in vivo observation of decreased IFN-γ levels in TRIF mutant mice by demonstrating that also under controlled conditions with equal amounts of growth-arrested bacteria the capacity of TRIF mutant splenocytes to secrete IFN-γ is impaired. Moreover, IFN-γ production was critically dependent on MyD88 and TLR4. This is not surprising, since it is well known that these innate immune sensors are highly important for the induction of the inflammatory response to *K. pneumoniae* and the phenotype of *Myd88*−/− and *Tlr4*−/− mice is more severe than that of TRIF mutant mice during in vivo infection (8, 25). However, the role of these receptors specifically in the induction IFN-γ in response to pathogens is less well known. In accordance with the present report, TRIF deficient mice were demonstrated to produce lower IFN-γ levels during *Aspergillus* airway infection in vivo (34). In the current study, our results suggest that TLR2 dependent signals play a role in response to *K. pneumoniae* in addition to TRIF, MyD88 and TLR4, since IFN-γ levels secreted by TRIF mutant and *Tlr4*−/− cells gradually increased with increasing bacterial concentrations, which is in line with the role of TLR2 during infection with *Klebsiella* in vivo (25).

We observed a spectacular effect on bacterial loads after reconstitution of TRIF mutant mice with rIFN-γ, which coincided with a partial recovery of the inflammatory cytokine profile. The importance of IFN-γ during *K. pneumoniae* infection was demonstrated before since *Ifn-γ*−/− mice displayed an impaired antibacterial defense and increased mortality (11, 12, 25). The other way around, in a rat model of ethanol intoxication followed by *Klebsiella* airway infection, adenoviral expression of IFN-γ improved antibacterial defense (35). Likewise, conditional adenoviral expression of IFN-γ improved clearance of *Klebsiella* from the lungs in mice (36). Strikingly, in our study there was no effect of rIFN-γ on bacterial loads in WT mice, suggesting that local rIFN-γ administration is only beneficial when it compensates for a clearly deficient production. Also, in WT mice rIFN-γ treatment, with the exception of IL-1β, did not affect lung cytokine concentrations, while in TRIF mutant mice it increased the levels of TNF-α, IL-6, CXCL1, CXCL2 and CCL2. The mechanism by which rIFN-γ improves bacterial defense in TRIF mutant mice might be by enhancing the
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bacterial killing capacity of alveolar macrophages (37). Unfortunately, the Klebsiella strain used here cannot be killed by macrophages or neutrophils in vitro (our own observations), illustrating its high virulence and precluding further in vitro analyses. Improved monocyte and macrophage function was also presumed to play a role in human clinical trials wherein treatment with rIFN-γ demonstrated beneficial effects in Mycobacterium (M.) tuberculosis and M. avium infections, Leishmaniasis and fungal sepsis, although the exact mechanisms are currently unknown (17-21). Recently, however, it was demonstrated in fungal sepsis patients that the ex vivo cytokine response was enhanced in patients treated with rIFN-γ (17). In our study, TRIF mutant mice treated with rIFN-γ also had higher plasma IFN-γ levels when compared with TRIF mutant mice treated with vehicle, even though rIFN-γ was instilled locally in the airways. Hence, although it is likely that the reduced bacterial loads at distant body sites in rIFN-γ treated TRIF mutant mice at least in part are the consequence of lower bacterial burdens at the primary site of infection, we cannot exclude an additional systemic effect of local rIFN-γ treatment. Another aspect of the inflammatory response that we observed in our study is that while total lung histopathology scores were not different between groups, rIFN-γ treated Trif-/- and WT mice had more signs of bronchitis and lower scores on pleuritis when compared to their respective vehicle treated controls, possibly indicating a redistribution in the pattern of inflammatory cell migration. This might be secondary to a higher intrabronchial rIFN-γ concentration after intranasal administration, resulting in increased attraction of inflammatory cells to the intrabronchial and intraalveolar compartment (see also figure 4). Possibly, this contributed to a better containment of the infection.

In this and in our previous study we demonstrated significantly lower levels of AST and ALT in mice (partially) deficient for TRIF, despite higher levels of bacterial loads in the blood and liver (8). Although liver bacterial loads were not determined in rIFN-γ treated mice, it is unlikely that the increased hepatocellular injury in these animals was caused by higher bacterial burdens in livers considering the reduced Klebsiella numbers in blood and spleen. This illustrates the double edged sword character of the innate immune response that is on the one hand essential for early antibacterial defense but on the other hand contributes to collateral tissue damage in sepsis as was illustrated in previous work (38-40). Strikingly, the reconstitution of TRIF mutant mice with rIFN-γ deteriorated liver injury. This suggests that IFN-γ is involved in inflammation driven liver injury, as was proposed before in an intravenous model of K. pneumoniae sepsis in Ifn-γ-/- mice (12). However, the lower AST levels in WT mice treated with rIFN-γ are more difficult to explain and require further investigation. Possibly, the increased plasma levels of the anti-inflammatory cytokine IL-10 in rIFN-γ treated WT mice (albeit not significant) played a role herein.

In conclusion, we demonstrate a crucial role for TRIF in IFN-γ production during K. pneumoniae pneumonia. TRIF mediated IFN-γ release is essential for an adequate innate immune response as reflected by the fact that the strongly impaired antibacterial defense of TRIF mutant mice can be restored by reconstitution of IFN-γ levels in the lungs by local treatment. These data provide new insight into
TIR-domain-containing adaptor-inducing interferon-β (TRIF) mediates antibacterial defense during gram-negative pneumonia by inducing Interferon-γ how TRIF mediates protective immunity during gram-negative infection.

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