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TIR-Domain-Containing Adaptor-Inducing Interferon-β (TRIF) Mediates Antibacterial Defense during Gram-Negative Pneumonia by Inducing Interferon-γ

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Key Words
Pneumonia · Toll-like receptors · Gram-negative sepsis

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 show here 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 adaptor 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 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 the 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 also contributes to TLR4 signaling, leading to the activation of

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NF-κB and interferon regulatory factor 3 (IRF3) and the induction of type I interferon (IFN) and inflammatory cytokine production [6]. Notably, TLR4, which recognizes lipopolysaccharide (LPS), activates the MyD88-dependent pathway before it initiates downstream signaling via the TRIF-dependent pathway once the TLR4 complex has been 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 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 (unpubl. 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, the upregulation of pathogen recognition, the processing and presentation of antigens and microbicidal effector cell functions [10]. A previous report, making use of IFN-γ gene deficient mice, demonstrated the importance of IFN-γ for antibacterial defense and survival during *K. pneumoniae* infection [11, 12]. In several models of experimental respiratory tract infection, it has been found that IFN-γ deficient mice are more susceptible to airway infection with *Legionella pneumophila* and *Burkholderia pseudomallei* [13, 14] and that therapeutic administration of recombinant (r)IFN-γ is beneficial [15, 16]. rIFN-γ also demonstrated a beneficial effect in several human studies when used as an adjunctive therapy for opportunistic pathogens [17–21].

We report here the impact of TRIF deficiency on pulmonary IFN-γ production during *Klebsiella* pneumonia. We explored to what 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, Tex., USA). 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 at 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). The 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 about 1×10^4 CFU of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, Va., USA) [8, 9]. The mice were sacrificed at the indicated time points after infection and their organs were harvested and processed exactly as described [8, 25]. In the reconstitution experiment, the mice were administered 50 ng of rIFN-γ (R&D Systems, Abbington, UK) or vehicle (0.1% human serum albumin in sterile saline) intranasally 30 min before and 24 h after inoculation; they were euthanized after 48 h 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 (q)PCR of the *Ifng* gene product was performed as described [26]. Data were analyzed using the LinRegPCR program. Results were normalized to the *β2m* transcript.

In vitro Studies

Splenocytes were obtained, seeded at a density of 500,000 cells per well and cultured exactly as described [9]. Cells were stimulated for 48 h 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* (100 ng/ml; Sigma) or ultrapure *Escherichia coli O111 B4* LPS (100 ng/ml; Invivogen) diluted in RPMI medium with antibiotics in a final volume of 200 μl. Supernatants were stored and analyzed for cytokine concentrations by ELISA.

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, Minn., USA and Invitrogen). 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 Hitachi analyzer (Boehringer Mannheim).

Histopathology

Histologic examination of lungs and liver was performed exactly as described [25, 27]. Granulocyte immunohistochemical staining was prepared using a FITC-labeled anti-mouse Ly6-C/G mAb (BD Biosciences, San Jose, Calif., USA) exactly as previously described [9].

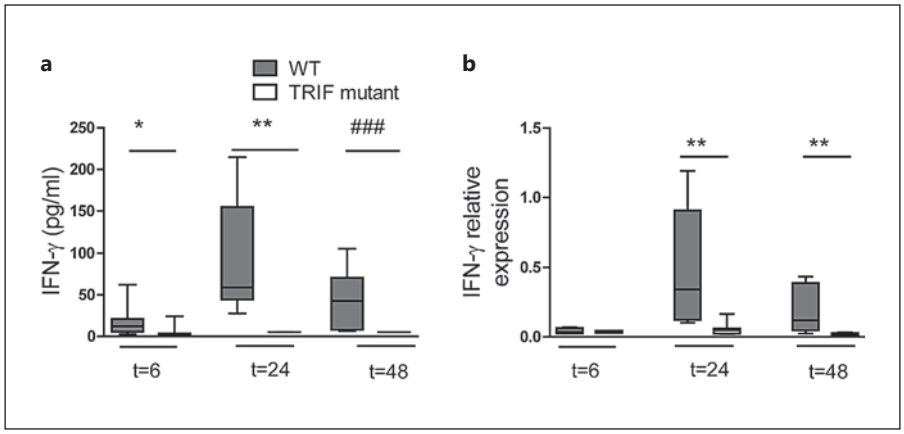

Fig. 1. TRIF mediates IFN-γ production during *K. pneumoniae* airway infection. WT and TRIF mutant mice (n = 7–8 per group) were infected with about 10^4 CFU *K. pneumoniae* and sacrificed at designated time points. IFN-γ levels in the lungs of the mice were determined by cytometric bead assay (**a**) and qRT-PCR (**b**). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. * $p < 0.05$, ** $p < 0.01$, Mann-Whitney U test. ### $p < 0.001$, Fisher exact test.

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 (SE) 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 Kruskal-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. The Fisher exact test was used to determine if the proportion of positive test results was different. These analyses were done using GraphPad Prism (San Diego, Calif., USA). $p < 0.05$ 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, illustrated by a significantly higher bacterial load in the lungs, blood and spleen [8]. In these investigations, we also observed higher bacterial loads in the livers of TRIF mutant mice and TRIF bone marrow chimeras lacking TRIF in hematopoietic cells (online suppl. fig. 1A, B; for all online suppl. material, see www.karger.com/doi/10.1159/000430913). In a multiplex cytokine assay performed on whole-lung homogenates, we noticed that IFN-γ levels remained undetectable in TRIF mutant mice throughout the infection (<5 pg/ml) whereas in WT mice, lung IFN-γ concentrations increased after *Klebsiella* inoculation, peaking after 24 h ($p < 0.05$–0.001 for the difference between groups; fig. 1a). TRIF mutant mice also showed strongly reduced IFN-γ mRNA expression in the lungs during *Klebsiella* pneumonia ($p < 0.01$ vs. WT mice; fig. 1b).

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 2×10^5 or 2×10^6 bacteria (data not shown). We repeated this experiment, this time including splenocytes of *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ mice in addition to those 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 ($p < 0.05$ to 0.01 compared to WT cells; fig. 2a). In addition, we stimulated cells with LPS derived from *K. pneumoniae* or ultrapurified LPS derived from *E. coli*, and found that IFN-γ release by *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$ and TRIF mutant cells was virtually absent ($p < 0.01$ vs. WT cells; fig. 2b).

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 contributed functionally to the impaired antibacterial defense of TRIF mutant mice, we treated WT and TRIF mutant mice with IFN-γ intranasally 30 min before and 24 h after infection with *Klebsiella*; we used 48 h of infection as a predefined end point, since this was the point at which the enhanced growth of *Klebsiella* in TRIF mutant relative to WT mice was clearest [8]. TRIF mutant mice treated with vehicle displayed undetectable pulmonary IFN-γ concen-

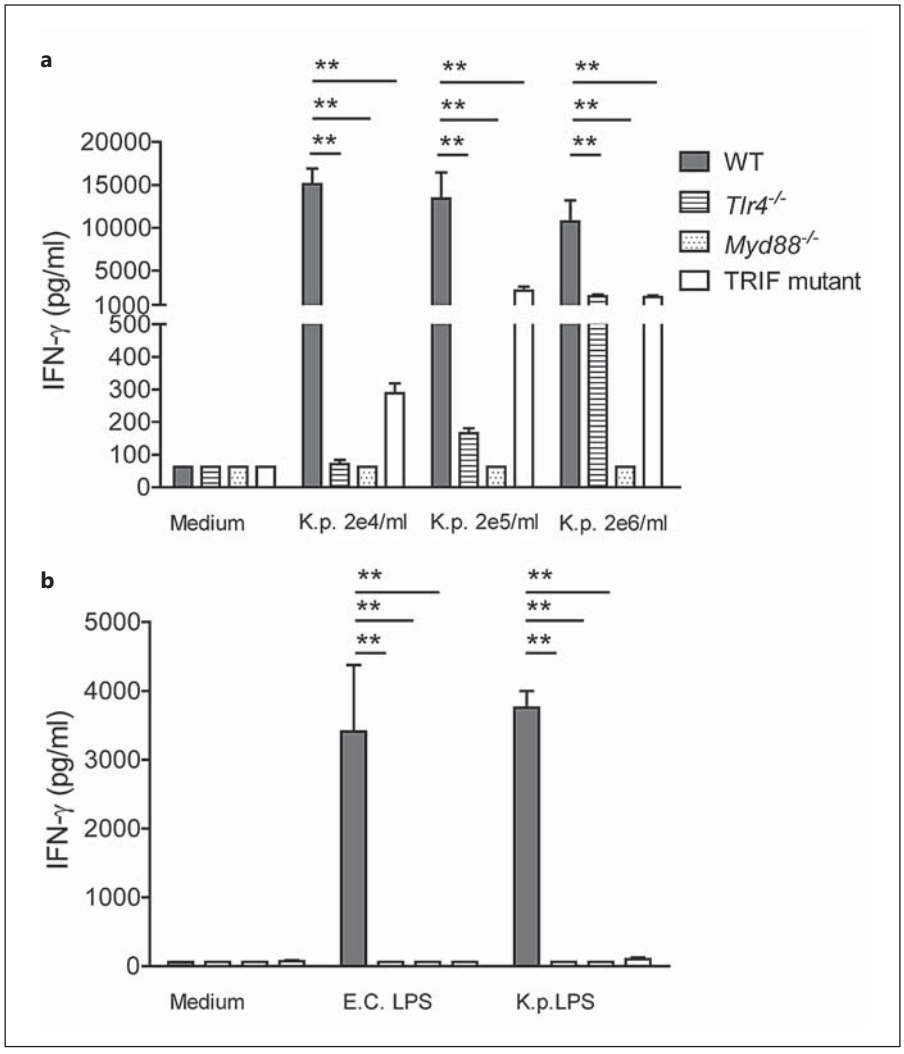

Fig. 2. IFN-γ secretion by splenocytes is dependent on TLR4, MyD88 and TRIF. Splenocytes derived from WT, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$ and TRIF mutant mice were stimulated with different concentrations of growth-arrested *K. pneumoniae* and LPS derived from *E. coli* or *K. pneumoniae* (n = 4–6 for each condition). IFN-γ levels were determined after 48 h. Data are expressed as mean (SE). ** $p < 0.01$, Mann-Whitney U test (performed post hoc after Kruskal-Wallis test).

trations, 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 (fig. 3a); WT mice that received rIFN-γ had significantly higher levels than WT mice treated with vehicle ($p < 0.05$). We reproduced the previously described phenotype in TRIF mutant mice [8], showing a 100- to 1,000-fold higher bacterial load in the lungs relative to WT mice, together with increased bacterial dissemination to the blood and spleen ($p < 0.001$; fig. 3b–d). Importantly, we observed a spectacular improvement of antibacterial defense in rIFN-γ-treated TRIF mutant mice compared to vehicle-treated TRIF mutant mice ($p < 0.01$–0.001), as reflected by bacterial loads similar to in 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 (fig. 3b–d).

Impact of IFN-γ Treatment on the Inflammatory Response to Pneumonia

To obtain insight into the extent of local inflammation at the primary site of infection in TRIF mutant and WT mice, and the effect on it of rIFN-γ treatment, we semi-quantitatively scored lung histopathology of tissue samples harvested 48 h after infection, focusing on key histological features characteristic for severe pneumonia. While total lung histopathology scores were not different between groups, rIFN-γ-treated TRIF mutant and WT mice had more signs of bronchitis and less signs of pleuritis than their respective vehicle-treated controls ($p < 0.05$ vs. controls; table 1; fig. 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 than in vehicle-treated

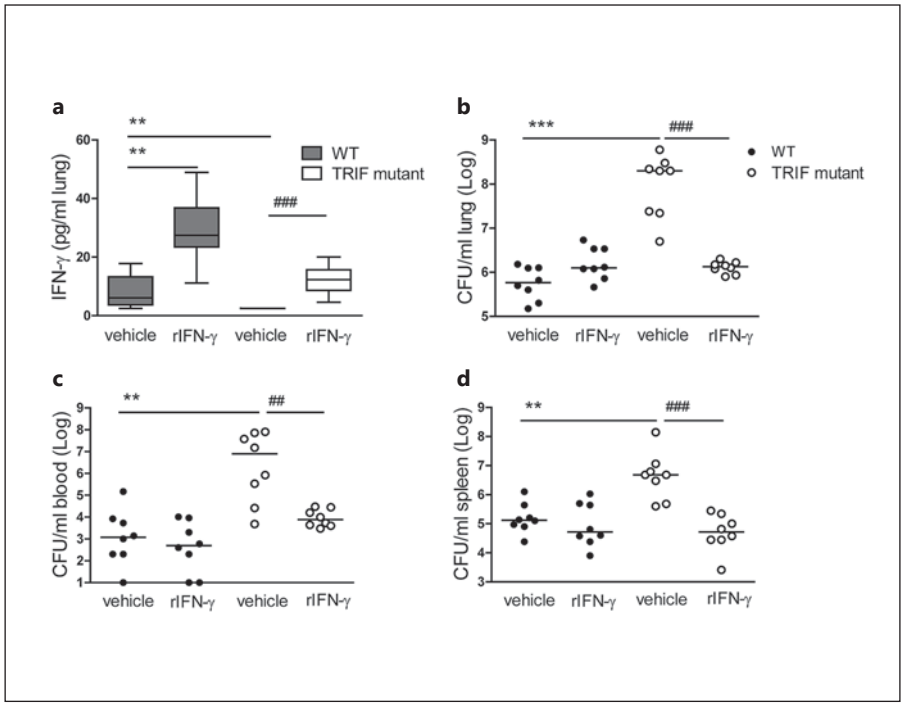

Fig. 3. Administration of rIFN-γ via the airways restores antibacterial defense in TRIF mutant mice. WT and TRIF mutant mice were infected with about 10^4 CFU *K. pneumonia*; 50 ng recombinant IFN-γ or vehicle was administered intranasally 30 min before infection and 24 h afterwards (n = 8 mice each group). Mice were sacrificed after 48 h of infection. IFN-γ levels in lung homogenates 48 h after infection (**a**) are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. Bacterial loads are shown in the lung (**b**), blood (**c**) and spleen (**d**) 48 h 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, Mann-Whitney U test, and Fisher exact test was used for comparison between TRIF mutant groups (performed post hoc after Kruskal-Wallis test).

Table 1. Histological scores: WT and TRIF mutant mice were infected with 1×10^4 CFU *K. pneumoniae* and 50 ng recombinant IFN-γ was administered intranasally upon infection and after 48 h

	WT vehicle	WT rIFN-γ	TRIF mutant vehicle	TRIF mutant rIFN-γ
Total pathology score: lung	14.5 (0.6)	13.8 (0.8)	14.5 (1.2)	13.1 (0.6)
Pneumonia % of lung surface	15 (3)	6 (4)	22 (6)	7 (3)
Interstitial inflammation	3.1 (0.1)	3.0 (0.5)	2.8 (0.7)	2.4 (0.3)
Edema	2.8 (0.2)	2.5 (0.3)	3.4 (0.5)	3.0 (0.2)
Endothelialitis	2.5 (0.2)	2.9 (0.1)	3 (0.2)	2.6 (0.2)
Bronchitis	2.9 (0.1)	3.5 (0.2)*	2.6 (0.3)	3.8 (0.2)#
Pleuritis	1.8 (0.3)	1.3 (0.2)*	1.5 (0.3)	0.8 (0.2)#
Ly-6-positive % of total lung surface	2.3 (0.4)	2.1 (0.5)	8.6 (1.2)**	3.9 (0.8)#

Total pathology score is the sum of the histological subscores (determined as described in Methods). Data are mean (SE) of 7–8 mice per group. * $p < 0.05$, ** $p < 0.01$, vs. vehicle-treated WT mice. # $p < 0.05$, rIFN-γ-treated TRIF mutant mice vs. vehicle-treated TRIF mutant mice.

WT mice at this late stage of infection ($p < 0.01$). 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). Moreover, when the Ly-6 staining was studied in detail, the number of intrabronchial neutrophils appeared to be greater after rIFN-γ treatment (fig. 4e–h). We next determined the effect of rIFN-γ treatment on the induction of the proinflammatory cytokines TNF-α, IL-1β and IL-6, the anti-inflammatory cytokine IL-10 and the 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 ($p < 0.05$–0.001; table 2). Treatment of TRIF mutant mice with

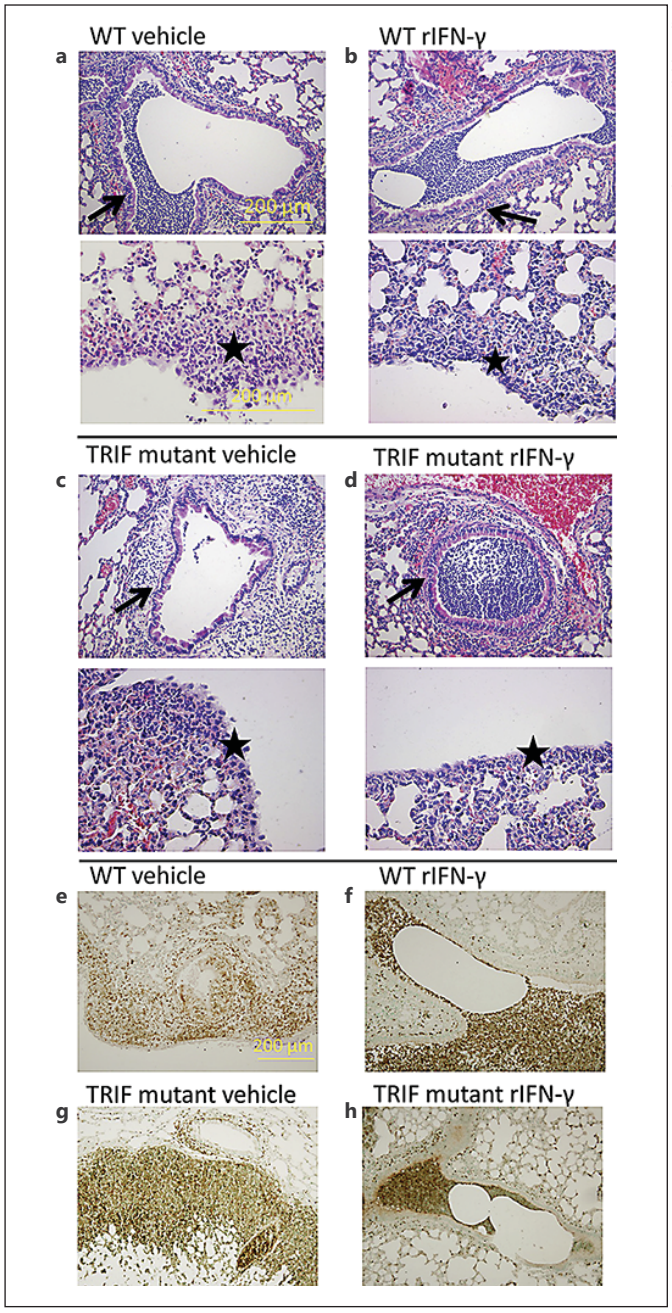

Fig. 4. Effect of IFN-γ treatment on lung pathology. WT and TRIF mutant mice were infected with about 10^4 CFU *K. pneumonia*; 50 ng recombinant IFN-γ or vehicle was administered intranasally 30 min before infection and 24 h afterwards. Mice were sacrificed after 48 h of infection. Representative lung histology 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**). HE. **a–d** Upper panel: arrows indicate signs of bronchitis. ×10. Lower panels: stars indicate pleuritis. ×20. 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**). ×10.

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 and the levels of CXCL1 and CCL2 were still significantly lower, although differences were smaller ($p < 0.05–0.01$ vs. 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 ($p < 0.05–0.001$ between groups; table 2).

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, 28]. TRIF mutant mice had lower AST and ALT plasma levels 48 h after infection than WT mice ($p < 0.01$; fig. 5a, b) as well as fewer signs of liver inflammation as determined by liver histopathology scores ($p < 0.01$; fig. 5c; online suppl. fig. 2). Remarkably, rIFN-γ treatment significantly increased the levels of AST and ALT in TRIF mutant mice compared to vehicle-treated TRIF mutant mice ($p < 0.01–0.001$), and these were similar to those measured in WT mice. In WT mice, rIFN-γ treatment reduced transaminase levels, significantly so for AST ($p < 0.05$; fig. 5a).

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, which limit therapeutic options [2–4, 29]. Previous research has documented the importance of TLR signaling for host defense during *K. pneumoniae* pneumonia, notably that of TLR4, TLR2 and TLR9 [25, 30, 31]. We and other study groups have previously described the pivotal role of the TLR-adaptors MyD88 and TRIF in this [8, 32]. 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 explored the functional importance here; our main findings were that TRIF is indeed 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,

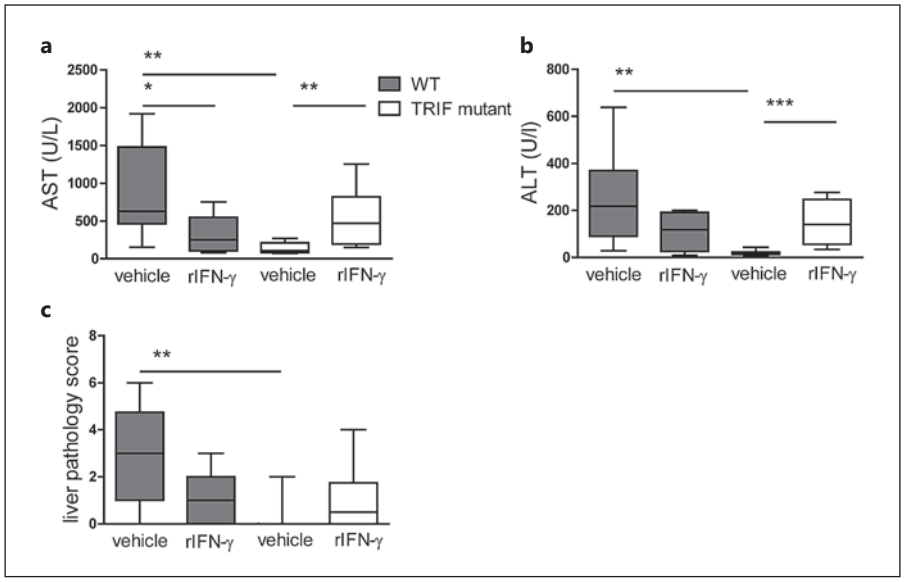

Fig. 5. TRIF mutant mice have attenuated liver injury that increases after rIFN-γ treatment. WT and TRIF mutant mice were infected with about 10^4 CFU *K. pneumonia;* 50 ng rIFN-γ or vehicle was administered intranasally 30 min before infection and 24 h afterwards. Mice were sacrificed after 48 h of infection. AST (**a**) and ALT (**b**) plasma levels and liver histopathology were scored (see Methods) (**c**) expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U test (performed post hoc after Kruskal-Wallis test).

Table 2. Inflammatory response

	WT vehicle	WT rIFN-γ	TRIF mutant vehicle	TRIF mutant rIFN-γ
TNF-α	892 (245)	760 (57)	191 (48)**	501 (115)$^{\#}$
IL-1β	7,434 (642)	4,950 (753)*	4,168 (731)**	4,525 (557)**
IL-6	1,914 (451)	2,030 (624)	2,446 (398)	1,507 (216)$^{\#}$
IL-10	14 (2)	11 (1)	14 (2)	b.d.
CXCL1	12,586 (1,899)	9,453 (1,645)	3,625 (871)**	4,255 (828)*
CXCL2	20,553 (6,546)	38,048 (7,157)	6,432 (1,532)*	28,943 (5,785)$^{\#\#\#}$
CCL2	4,619 (541)	3,718 (366)	1,841 (210)***	2,126 (240)**, $^{\#}$

WT and TRIF mutant mice were infected with 1×10^4 CFU *K. pneumoniae* and 50 ng recombinant IFN-γ was administered intranasally upon infection and after 48 h. Homogenates were prepared from right lungs. Cytokine and chemokine levels are presented in pg/ml of lung homogenate. Data are mean (SE) of 7–8 mice per group. b.d. = Below detection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. vehicle-treated WT mice. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, rIFN-γ-treated TRIF mutant mice vs. vehicle-treated TRIF mutant mice.

marked by a clearly impaired antibacterial defense with a 100- to 1,000-fold increase in bacterial load 48 h after infection, a finding that we have reproduced here [8, 25, 32]. The early inflammatory response of mice that are 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 a higher bacterial load, all of these cytokines gradually increased in spite of (partial) TRIF deficiency [8]. Notably, in this study, CXCL1, CXCL2 and TNF-α levels were still reduced in TRIF mutant mice 48 h after 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 time point, which leads to tissue injury and neutrophil attraction via mechanisms apart from what is provided by the chemoattractant gradient by the aforementioned mediators. Remarkably, however, IFN-γ levels remained virtually undetectable in TRIF mutant mice throughout, which formed the rationale for our 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, processing and presentation of antigens 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 that 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 agreement with this report, TRIF-deficient mice have been shown to produce lower IFN-γ levels during *Aspergillus* airway infection in vivo [33]. 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 along 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 load after the 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 has been demonstrated previously, 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 [34]. Likewise, conditional adenoviral expression of IFN-γ improved clearance of *Klebsiella* from the lungs in mice [35]. 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. In addition, in WT mice, rIFN-γ treatment did not affect lung cytokine concentrations (with the exception of IL-1β) whereas 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 bacterial killing capacity of alveolar macrophages [36]. 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 where treatment with rIFN-γ demonstrated beneficial effects in *Mycobacterium tuberculosis* and *M. avium* infections as well as with leishmaniasis and fungal sepsis, although the exact mechanisms are currently unknown [17–21]. Recently, however, in fungal sepsis patients, it was demonstrated 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 than 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 did not differ between groups, rIFN-γ-treated *Trif*$^{-/-}$ and WT mice had more signs of bronchitis and lower pleuritis scores 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 an increased attraction of inflammatory cells to the intrabronchial and intra-alveolar compartment (fig. 4). Possibly, this contributed to 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 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 the liver, considering the reduced *Klebsiella* numbers in the 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, contributes to collateral tissue damage in sepsis as illustrated in previous studies [37–39]. Strikingly, the reconstitution of TRIF mutant mice with rIFN-γ caused 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]. How-

ever, 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 here.

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 the reconstitution of IFN-γ levels in the lungs by local treatment. These data provide new insight into how TRIF mediates protective immunity during Gram-negative infection.

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