Cell-specific pattern recognition receptor signaling in antibacterial defense
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Chapter 3

Differential roles of MyD88 and TRIF in hematopoietic and resident cells during murine gram-negative pneumonia

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Abstract

Background:
Pneumonia is frequently caused by gram-negative pathogens, among which *Klebsiella pneumoniae* prominently features. Recognition of pathogen-associated molecular patterns by Toll-like receptors (TLRs) is important for an appropriate immune response during infection. TLR signaling can proceed via two distinct routes which are dependent on the adaptor proteins Myeloid differentiation primary response gene (88) (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF) respectively. Aim of the study was to determine the relative contribution of MyD88 and TRIF signaling in resident and hematopoietic cells to host defense during pneumonia.

Methods:
Bone marrow chimeras of MyD88 deficient/wild type and TRIF mutant/wild type mice were created and infected with *K. pneumoniae* via the airways.

Main results:
MyD88 in both resident and hematopoietic cells contributed to survival and antibacterial defense in late stage infection, whereas only TRIF in hematopoietic cells was protective. On the other hand, resident MyD88 and hematopoietic TRIF contributed to distant cellular injury. Resident MyD88 was pivotal for early chemokine release and neutrophil recruitment in the bronchoalveolar space.

Conclusion:
MyD88 and TRIF dependent signaling have a differential contribution to host defense in different cell types that changes from early to late stage gram-negative pneumonia.
Introduction

Pneumonia is the most common cause of sepsis. Lower respiratory tract infections are frequently caused by gram-negative pathogens, including *Klebsiella pneumoniae* (1,2). Emerging microbial resistance amongst *Enterobacteriaceae* is an issue of major concern, limiting therapeutic options and increasing mortality rates (3). Toll-like receptors (TLRs) occupy a prominent position in the innate immune system by virtue of their capacity to recognize bacterial components (4, 5). TLR signaling can proceed via two distinct routes which are dependent on myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF) (4, 5). MyD88 is the universal adaptor for all TLRs except TLR3; TRIF is the sole adaptor for TLR3, and in addition contributes to TLR4 signaling. Very recently, mice deficient for either MyD88 or TRIF were found to be more susceptible to death after infection with *Klebsiella* via the airways, which was accompanied by enhanced bacterial growth in both mouse strains (6). Several MyD88-dependent TLRs likely contribute to the susceptible phenotype of MyD88 deficient mice during *Klebsiella pneumonia*, in particular TLR4 and TLR9, whereas TLR2 may contribute to host defense during late stage infection (7-10). Other MyD88 dependent TLR's have not been studied in models of *Klebsiella* infection, but are less likely to be involved considering their specificity for pathogen ligands that are not expressed by this bacterium.

TLRs are expressed by both hematopoietic and resident cells and both cell types contribute to an effective host defense during respiratory tract infections (11-13). We here aimed to investigate the cell-type specific role of MyD88 and TRIF during early and late stage *Klebsiella* infection, by creating bone marrow chimeras expressing MyD88 or TRIF only in radioresistant (resident, R) cells or radiosensitive (hematopoietic, H) cells.

Methods

**Animals**

MyD88 gene deficient (*Myd88*⁻⁻) mice were provided by Dr. S. Akira (Research Institute for Microbial Diseases, Osaka, Japan) and backcrossed > 6 times to a C57Bl/6 genetic background (14). TRIF mutant mice, generated on a C57Bl/6 genetic background (15), were provided by Dr B. Beutler (the Scripps Research Institute, La Jolla, CA). Age- and sex matched wild-type (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 7x10³ colony forming units (CFU) of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA) (9, 16). Mice were followed for a maximum of 14 days or in separate experiments euthanized at 6, 24 or 48 hours after infection mice after which organs were harvested and processed for the determination of
bacterial outgrowth and cytokine levels as described (9). In some experiments bronchoalveolar lavage (BAL) was performed and cell counts determined in BAL fluid (BALF) (17).

Assays
TNF-α, IL-6, IL-10 and MCP-1 (monocyte chemotactic protein 1, also known as CCL2) were measured by using a cytometric bead array multiplex assay (BD Biosciences, San Jose, CA). Cytokine-induced neutrophil chemoattractant (KC, also known as CXCL1), MIP-2 (Macrophage inflammatory protein 2 alpha, also known as CXCL2), LPS-induced CXC chemokine (LIX) and E-selectin were measured by ELISA's (R&D Systems, Minneapolis, MN). Lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine transaminase (ALT) were measured with kits from Sigma (St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Bone marrow transplantation
Bone marrow chimeric mice were generated as described (9, 18, 19). Briefly, recipient groups received a lethal total body irradiation of two times 4.5 Gy with three hours between the two doses, using a 137Cs irradiator (CIS Bio International, Gif, France) at a dose rate of 0.5 Gy/min, followed by intravenous injection of 5x10^6 bone marrow cells that were isolated from donor animals as described before (9). To protect the irradiated recipient mice from immediate infections, the mice were also injected with 2x10^5 splenocytes from donor animals that were crushed through 40 μm filter, washed and resuspended in PBS. Moreover, mice were provided with autoclaved, acidified drinking water containing 0.16% neomycin sulfate (Sigma Chemical Co. St.Louis, MO) from one week before until four weeks after transplantation. Pneumonia was induced 6 weeks after transplantation. Engraftment was checked by flow cytometry based on differential expression of CD45.1 and CD45.2 by donor and recipient cells exactly as described (9,19). As a control for the transplantation procedure, we not only administered TRIF mutant or Myd88^-/- bone marrow cells (H-) into WT recipient mice (R+) and WT bone marrow cells (H+) into TRIF mutant or Myd88^-/- recipient mice (R-), but also WT bone marrow (H+) to WT mice (R+) and TRIF mutant or Myd88^-/- bone marrow (H-) to TRIF mutant or Myd88^-/- mice (R-) respectively. Thus, in each experiment with chimeric mice four groups of mice were generated (R-/H+, R+/H- and as controls R+/H+ and R-/H-).

Statistical analysis
Data are expressed as means ± standard error of the mean; as medians with individual data points (for bacterial loads); or as Kaplan-Meier plots. For experiments with two groups Mann Whitney U test was used to determine significance. For experiments with more than two groups Kruskall-Wallis test was used as a pretest, in order to reduce the chance of committing a type 1 error. When appropriate, Mann Whitney U tests were used as follow-up tests to compare individual genetically modified groups to the R+/H+ control group. Survival curves were compared using log-rank test. All analyses were done using GraphPad Prism (San Diego, CA). p < 0.05 was considered statistically significant.
Results

Both MyD88 and TRIF are crucial for survival and the antibacterial response in gram-negative pneumonia

We first infected Myd88⁻/⁻, TRIF mutant and WT mice with *K. pneumoniae* via the airways and followed them for 10 days in two independent survival experiments (Figure 1A and D). All Myd88⁻/⁻ mice died before 48 hours, while 50% of WT mice remained alive until the end of the experiment (*p* < 0.001). TRIF mutant mice also showed a higher and accelerated mortality compared to WT mice (*p* < 0.01), although lethality did not occur as rapidly as in Myd88⁻/⁻ mice.

Next, we euthanized Myd88⁻/⁻, TRIF mutant and WT mice at predefined time points for quantitative cultures of lungs, blood and spleen. In experiments comparing Myd88⁻/⁻ and WT mice, these analyses were confined to the first 24 hours considering the large number of deaths amongst Myd88⁻/⁻ mice thereafter. At 6 hours, the bacterial loads in the lungs of both Myd88⁻/⁻ and TRIF mutant mice were similar to those in WT mice and cultures of blood and spleen remained sterile in all but one Myd88⁻/⁻ mouse. After 24 hours, Myd88⁻/⁻ mice had about 2-log more bacteria in their lungs when compared to WT mice (Figure 1B, *p* < 0.01), while dissemination to blood (not shown) and spleen (Figure 1C) was not different. At 48 (but not 24 hours), TRIF mutant mice had remarkably higher bacterial burden in their lungs (Figure 1E), as well as in blood (not shown) and spleen (Figure 1F, *p* < 0.001 versus WT mice). The fact that TRIF mutant mice showed enhanced bacterial loads in their spleens while Myd88⁻/⁻ mice did not, is likely explained by the different durations of the infection at the time of euthanasia (48 versus 24 hours respectively). These data confirmed the essential role of MyD88 and TRIF in host defense during *Klebsiella pneumonia* (6) and further show that the role of TRIF in protective immunity becomes apparent later in the course of the infection in comparison to MyD88.

MyD88 expression in both hematopoietic and resident cells contributes to survival, while hematopoietic TRIF expression is most important for survival

To dissect the role of MyD88 and TRIF dependent TLR signaling in hematopoietic (H) versus resident (R) cells, we created bone marrow chimeric mice for MyD88 and TRIF. In accordance with our earlier reports (9, 18, 19), the mean percentage of donor derived neutrophils and monocytes in blood from all chimeric mice was >90% (data not shown). MyD88 R⁻/H⁻ mice displayed a strongly accelerated mortality when compared with MyD88 H⁺/R⁺ mice (*p* < 0.0001; Figure 2A). Of more interest, MyD88 R⁺/H⁻ mice and MyD88 R⁻/H⁺ mice also demonstrated an accelerated mortality when compared with MyD88 R⁺/H⁺ mice (both *p* < 0.001), whereas the mortalities amongst both chimeric MyD88 strains (R⁺/H⁻ and R⁻/H⁺) were significantly delayed when compared with MyD88 R⁻/H⁻ mice (*p* < 0.001 and *p* <0.05 respectively). Mortality curves of MyD88 R⁺/H⁻ and R⁻/H⁺ mice were not different. TRIF R⁻/H⁻ mice displayed a strongly accelerated mortality when compared with
Figure 1: MyD88 and TRIF protect against lethality and restrict bacterial growth in gram negative pneumonia. WT, Myd88−/− and TRIF mutant mice were inoculated with 7x10^3 CFU K. pneumoniae and monitored for survival or sacrificed at designated time-points. Survival of WT (closed squares) and Myd88−/− (open rounds) mice (n=8 per group) (A). Bacterial loads in lung (B) and spleen (C) 6 and 24 hours after infection in WT (closed squares) and Myd88−/− mice (open rounds). Survival of WT (closed squares) and TRIF mutant mice (open rounds) (n=17 per group) (D). Bacterial loads in lung (E) and spleen (F) 6, 24 or 48 hours after infection in WT (closed squares) or TRIF mutant mice (open rounds) (n=7-8 per group). Each symbol represents an individual mouse, with horizontal lines showing medians. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to WT mice determined with Mann-Whitney U test.

TRIF R+/H+ mice (p < 0.0001, Figure 2B). TRIF R+/H- mice and TRIF R-/H+ mice both showed an accelerated mortality when compared with TRIF R+/H+ mice (both p < 0.01), but there was no significant difference between the mortality curves of TRIF R-/H+ mice and TRIF R+/H- mice. Importantly, the mortalities amongst TRIF R-/H+ mice were delayed when compared with TRIF R-/H- mice (p < 0.05) while the survival of TRIF R+/H- mice was not significantly different from TRIF R-/H- mice.

MyD88 expression in both hematopoietic and resident cells limits bacterial growth, whereas hematopoietic but not resident TRIF expression reduces bacterial multiplication

We next infected MyD88 and TRIF chimeric mice with Klebsiella via the airways and euthanized them at 24 (MyD88 chimeras) or 48 hours (TRIF chimeras), i.e. at time points that had revealed the importance of MyD88 and TRIF in reducing bacterial growth in mice with a general deficiency for these adaptor proteins. As expected, MyD88 R-/H- and TRIF R-/H- mice demonstrated enhanced bacterial outgrowth in their lungs at 24 and 48 hours after infection respectively when compared to their respective R+/H+ controls (Figure 3A and D). Of considerable
interest, 24 hours after infection not only MyD88 R-/H- mice showed enhanced bacterial growth in lungs and distant body sites (blood and spleen), but also mice deficient for MyD88 in either hematopoietic or resident cells had increased bacterial loads in all body compartments when compared with MyD88 H+/R+ mice (Figure 3A-C). The difference between MyD88 R+/H- and R+/H+ mice was larger than between MyD88 R-/H+ and R+/H+ mice, indicating that MyD88 signaling in resident cells does contribute to host defense but that the expression of MyD88 in hematopoietic cells is even more important. In contrast, the experiments with TRIF chimeras only revealed a role for TRIF expressed in hematopoietic cells in reducing bacterial growth and dissemination. Indeed, at 48 hours after infection TRIF R+/H- mice, but not TRIF R-/H+ mice, displayed higher bacterial burdens in lungs, blood and spleen when compared with TRIF R+/H+ mice; in addition, bacterial loads in TRIF R+/H- mice were similar to those in TRIF R-/H- mice in all body compartments (Figure 3D-F).
Limited role of MyD88 or TRIF in lung cytokine and chemokine production during late stage infection

We measured the lung concentrations of the proinflammatory cytokines TNF-α, IL-6, the anti-inflammatory cytokine IL-10 and the chemokines KC, MIP-2 and MCP-1 at 24 hours (MyD88 chimeric mice) or 48 hours (TRIF chimeric mice) after infection. The levels of all mediators showed a large variation amongst groups and differences between groups were modest at best (Supplementary tables 1 and 2). These data suggest that neither MyD88 nor TRIF signaling is essential for the production of these mediators in the lungs during late stage pneumonia.

Figure 3: MyD88 expression in both resident and hematopoietic cells limits bacterial growth, whereas hematopoietic but not resident TRIF expression reduces bacterial multiplication. WT (R+) and Myd88−/− or TRIF mutant (R−) mice were irradiated and injected with WT (H+), Myd88−/− or TRIF mutant (H−) bone marrow cells. Six weeks after transplantation, mice were infected with 7x10^3 CFU K. pneumoniae. Bacterial loads in lung (A), blood (B) and spleen (C) of MyD88 chimeras 24 hours after infection (n = 8-14 per group). Bacterial loads in lung (D), blood (E) and spleen (F) of TRIF mutant chimeras 48 hours after infection (n = 9-11 per group). Each symbol represents an individual mouse, with horizontal lines showing medians.* p < 0.05, ** p < 0.01, *** p < 0.001 vs R+/H+ mice determined with Mann-Whitney U test as a follow-up test on Kruskall-Wallis test.
Resident MyD88 and hematopoietic TRIF expression contribute to distant organ injury in late stage infection

The model of gram-negative pneumonia used here is associated with rises in the plasma concentrations of LDH (indicative for cellular injury in general) and AST/ALT (reflecting hepatocellular injury) in the late stage of infection (16). Remarkably, none of the partially or fully MyD88 deficient mice demonstrated evidence for enhanced cell injury when compared with MyD88 R+/H+ mice (Figure 4): plasma LDH and AST concentrations were even slightly lower in MyD88 R-/H+ mice ($p < 0.05$ versus MyD88 R+/H+ mice), suggesting that MyD88 in resident cells contributes to cell injury during Klebsiella infection (Figure 4A-C). In TRIF chimeras there was a distinct contribution of TRIF in hematopoietic cells to cellular injury: TRIF R+/H- mice and TRIF R-/H- mice had significantly lower plasma LDH, AST and ALT values when compared to TRIF R+/H+ or TRIF R-/H+ mice ($p < 0.05$ to $p < 0.001$, Figure 4D-F). On the other hand, the plasma levels of cellular injury markers were not different between TRIF R+/H+ and TRIF R-/H+ mice.

Differential contribution of MyD88 and TRIF in hematopoietic and resident cells in early stage infection

We repeated the experiments in MyD88 and TRIF chimeras, this time sacrificing the mice after 6 hours of infection (Figure 5). This early time point was not chosen to determine the impact of cell-specific MyD88 or TRIF deficiency on bacterial growth, but rather to establish their role in induction of an early innate immune response. MyD88 R-/H+ mice had a slightly higher bacterial burden in their lungs when compared with MyD88 R+/H+ mice ($p < 0.05$), whereas both TRIF R-/H+ and TRIF R-/H- mice displayed modestly elevated bacterial counts when compared with TRIF R+/H+ mice ($p < 0.05$). Blood cultures were sterile in all chimeric animals. Both hematopoietic and resident MyD88 and TRIF contributed to recruitment of neutrophils, although with different relative importance.

Indeed, MyD88 R-/H+ and R-/H- mice showed a similar dramatic reduction in neutrophil influx into BALF when compared with MyD88 R+/H+ mice (both $p < 0.001$), whereas MyD88 R+/H- demonstrated an intermediate phenotype ($p < 0.01$ versus MyD88 R+/H+ mice and $p < 0.001$ versus both MyD88 R-/H- and R-/H+ mice; Figure 6A), indicating that especially MyD88 in resident cells drives the early neutrophil migration into the alveolar space during Klebsiella pneumonia. On the other hand, hematopoietic and resident TRIF appeared to be equally important: relative to TRIF R+/H+ mice, TRIF R+/H-, R-/H+ and R-/H- mice all had similarly reduced neutrophil numbers in BALF (all $p < 0.05$ versus TRIF R+/H+ mice; Figure 6E). Consistent with the more prominent role for resident MyD88 in neutrophil recruitment, the BALF levels of neutrophil attracting chemokines KC, MIP-2 and LIX were especially reduced in MyD88 R-/H+ mice (Figure 6B-D). With regard to KC and LIX, MyD88 R-/H+ and R-/H- mice demonstrated similarly reduced BALF levels when compared with MyD88 R+/H+ mice; MIP-2 levels were even only diminished in MyD88 R-/H+ mice. In contrast, BALF MIP-2 and LIX concentrations were similar in all TRIF chimeras,
Figure 4: Resident MyD88 and hematopoietic TRIF contribute to distant organ injury. WT (R+) and Myd88−/− or TRIF mutant (R-) mice were irradiated and injected with WT (H+), Myd88−/− or TRIF mutant (H-) bone marrow cells. Six weeks after transplantation, mice were infected with 7x10³ CFU K. pneumoniae. Plasma levels of LDH (A), AST (B) and ALT (C) in MyD88 chimeras 24 hours after infection (n = 8-14 per group). Plasma levels of LDH (D), AST (E) and ALT (F) in TRIF mutant chimeras 48 hours after infection (n = 9-11 per group). Bars represent mean ± standard error of the mean. * p < 0.05, ** p < 0.01, *** p < 0.001 vs R+/H+ mice determined with Mann-Whitney U test as a follow-up test on Kruskall-Wallis test.

Figure 5: Bacterial loads in early stage infection in mice chimeric for MyD88 or TRIF. WT (R+) and Myd88−/− or TRIF mutant (R-) mice were irradiated and injected with WT (H+) or Myd88−/−/TRIF mutant (H-) bone marrow cells. Six weeks after transplantation, mice were infected with 7x10³ CFU K. pneumoniae and sacrificed 6 hours later (n = 8-12 per group). Bacterial loads in lung homogenates of MyD88 chimeras (A) and of TRIF mutant chimeras (B). Each symbol represents an individual mouse, with horizontal lines showing medians. * p < 0.05, ** p < 0.01 vs R+/H+ mice determined with Mann-Whitney U test as a follow-up test on Kruskall-Wallis test.
Figure 6: Differential contribution of MyD88 and TRIF in resident and hematopoietic cells to the host response in early stage infection. WT (R+) and Myd88−/− or TRIF mutant (R−) mice were irradiated and injected with WT (H+) or Myd88−/− TRIF mutant (H−) bone marrow cells. Six weeks after transplantation, mice were infected with 7x10⁵ CFU K. pneumoniae and sacrificed 6 hours later (n = 8-12 per group). Number of neutrophils (A) and levels of KC (B), MIP-2 (C) and LIX (D) in BALF of MyD88 chimeras. Number of neutrophils (E) and levels of KC (F), MIP-2 (G) and LIX (H) in BALF of TRIF mutant chimeras. Bars represent mean ± standard error of the mean.* p < 0.05, ** p < 0.01, *** p < 0.001 vs R+/H+ mice determined with Mann-Whitney U test as a follow-up test on Kruskall-Wallis test.
whereas KC levels were reduced in mice lacking TRIF in either hematopoietic or resident cells or both (Figures 6F-H).

Resident MyD88 clearly also was most important for the early release of proinflammatory cytokines into BALF: TNF-α and IL-6 levels were equally reduced in MyD88 R-/H+ and R-/H- mice (Figure 7A and B). In contrast, hematopoietic TRIF determined TNF-α and IL-6 release: TRIF R+/H- and R-/H- mice displayed equally reduced BALF TNF-α and IL-6 concentrations when compared with either TRIF R+/H+ or TRIF R-/H+ mice (Figure 7D and E). In addition, resident MyD88 was most important for the upregulation of E-selectin (a marker for endothelial cell activation) in the lungs: MyD88 R-/H+ and R-/H- mice had significantly lower lung E-selectin levels when compared to MyD88 R+/H+ mice ($p < 0.05$ and $p < 0.001$; Figure 7C). The absence of TRIF in neither resident nor hematopoietic cells influenced lung E-selectin levels (Figure 7F).

Figure 7: Levels of cytokines in BALF and levels of E-selectin in lung homogenates in early stage infection in mice chimeric for MyD88 or TRIF. WT (R+) and Myd88−/− or TRIF mutant (R-) mice were irradiated and injected with WT (H+) or Myd88−/− TRIF mutant (H-) bone marrow cells. Six weeks after transplantation, mice were infected with $7 \times 10^3$ CFU K. pneumoniae and sacrificed 6 hours later (n = 8-12 per group). Levels of TNF-α (A) and IL-6 (B) in BALF and levels of E-selectin in lung homogenate (C) of MyD88 chimeras. Levels of TNF-α (D) and IL-6 (E) in BALF and levels of E-selectin in lung homogenate (F) of TRIF mutant chimeras. Bars represent mean ± standard error of the mean.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs R+/H+ mice determined with Mann-Whitney U test as a follow-up test on Kruskall-Wallis test.
Discussion

MyD88 is the common adaptor for all TLRs (except TLR3) and deficiency of this proximal protein in TLR signaling has recently been demonstrated to result in a strongly impaired host defense during respiratory tract infection by K. pneumoniae (6). Deficiency of TRIF, which in addition to MyD88 is responsible for cellular activation by TLR4, also was associated with a hypersusceptible phenotype during Klebsiella pneumonia (6). We here show that MyD88 induced protection during Klebsiella pneumonia and sepsis is mediated by both hematopoietic and resident cells, while TRIF mediated protection is primarily driven by hematopoietic cells. These cell-specific protective functions of MyD88 and TRIF corresponded with their role in limiting bacterial growth, but not with the extent of cellular injury in distant organs, as measured by the plasma concentrations of AST, ALT and LDH. Indeed, our results indicate that MyD88 in resident cells and TRIF in hematopoietic cells contributed to cell injury during late stage infection. Hence, these findings suggest that in the present model mortality likely occurs as a consequence of excessive bacterial growth and nicely illustrate the “double edged sword” character of innate immune activation via MyD88 and TRIF dependent signaling.

The role of hematopoietic and resident MyD88 has been studied previously in murine Pseudomonas aeruginosa pneumonia (20). Herein, mice expressing MyD88 only in resident cells cleared the pathogen equally well as their controls with intact MyD88 expression (20). Accordingly, selective expression of MyD88 in lung epithelial cells was sufficient for clearance of Pseudomonas from the lungs (21). Notably, the Pseudomonas pneumonia model in mice differs considerably from the Klebsiella model used here; the current model more closely resembles the clinical scenario of a gradually growing bacterial load (22).

We here demonstrate that MyD88 expressed by hematopoietic and resident cells is involved in early (< 6 hours) influx of neutrophils during Klebsiella pneumonia, but that clearly resident MyD88 plays the more prominent part. The importance of MyD88 dependent signaling in resident cells for the attraction of neutrophils in mice chimeric for MyD88 was reported previously in a model of Pseudomonas pneumonia (20). Accordingly, selective expression of MyD88 in lung epithelial cells was sufficient for neutrophil attraction during Pseudomonas pneumonia (21). TRIF signaling also contributed to neutrophil attraction into BALF, wherein hematopoietic and resident TRIF seemed to be of similar importance. The reduced BALF CXC chemokine levels in MyD88 and TRIF chimeras likely contributed to this attenuated neutrophil migration (23-25).

Potential radioresistant cell populations that contribute to protective TLR signaling during Klebsiella pneumonia include epithelial, endothelial and stromal cells. Amongst these, in particular respiratory epithelial cells have been implicated to play an important role in the early phase of infection by virtue of their capacity to release an array of antimicrobial peptides and to secrete chemokines that orchestrate the recruitment of neutrophils to the alveolar space (13). Airway epithelial cells
especially express TLR2-6 (13), strategically positioned to enable immediate recognition of organisms entering the airways.

Several MyD88 dependent receptors can contribute to the hypersusceptible phenotype of MyD88 deficient mice: MyD88 is not only the adaptor for multiple TLRs but also for the IL-1 and IL-18 receptors. MyD88 dependent receptors contributing to host defense in *Klebsiella* pneumonia include TLR2, TLR4 and TLR9 (7-10); 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 (26). The phenotype of (bone marrow chimeric) TLR2/4 double KO mice was remarkably similar to that of (bone marrow chimeric) Myd88-/- mice, suggesting that TLR2 and TLR4 are the most important MyD88 dependent receptors involved during *Klebsiella* infection (9). The protective role of hematopoietic TRIF reported here most likely is mediated via TLR4, considering that mice deficient for TLR3 (which relies exclusively on TRIF for signaling) demonstrate similar bacterial loads during *Klebsiella* pneumonia when compared with WT mice (our own unpublished data). Of note, a subset of macrophages harvested from TRIF mutant mice were reported to still respond to LPS, most likely via the TRAM adapter (15). Considering the strong phenotype of TRIF mutant mice shown here, it is likely that this pathway does not contribute significantly to protective immunity during *Klebsiella* infection.

Our observations are in contrast with the only other report that studied the relative role of TRIF in a mouse bone marrow chimera model in a lung infection model: there the TLR3-TRIF dependent axis in resident cells was shown to be crucial for an effective host response against *Aspergillus*, by balancing the Th1 and Th17 response (27). Clearly, the pathogenesis and defense mechanisms that lead to a beneficial outcome during aspergillosis are different from these processes during *Klebsiella* infection.

In conclusion, we here document for the first time to our knowledge the relative importance of the essential TLR adaptors MyD88 and TRIF in different cell types and how their contribution changes during early and late stage infection. Our results provide new insights in the pathophysiology of *Klebsiella* pneumonia and the potential of therapeutic targeting of TLR dependent pathways.

### Acknowledgements

The authors thank Dr. S. Akira (Research Institute for Microbial Diseases, Osaka, Japan) and Dr. B. Beutler (The Scripps Research Institute, La Jolla, CA) for providing the Myd88-/- and TRIF mutant mice respectively. The authors thank Joost Daalhuisen, Marieke ten Brink and Hans Roodermond for excellent technical assistance.
Differential role of MyD88 and TRIF signaling in pneumonia

References


Chapter 3


## Supplementary appendix chapter 3

Supplementary Table 1: Lung cytokine levels in MyD88 chimeric mice.

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Mice chimeric for MyD88 were infected with $7 \times 10^3$ CFU *K. pneumonia* 6 weeks after bone marrow transplantation. Twenty-four hours after infection, mice were sacrificed, lungs were removed and cytokine levels determined in lung homogenates. KC and MIP-2 were determined using ELISA. TNF-α, IL-6, IL-10 and MCP-1 levels were determined by Cytometric Bead Assay. Data are presented in pg/ml lung homogenate as mean ± SEM. N=8-14 mice per group. Bd= below detection level. *p < 0.05, **p < 0.01, ***p < 0.001 vs R+/H+ levels determined with Mann Whitney U test. R recipient and H hematopoietic.
Supplementary Table 2: Lung cytokine levels in TRIF chimeric mice.

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<th>R+/H-</th>
<th>R-/H-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>196 ± 71</td>
<td>156 ± 39</td>
<td>241 ± 68</td>
<td>328 ± 240</td>
</tr>
<tr>
<td>IL-6</td>
<td>1195 ± 760</td>
<td>1701 ± 509</td>
<td>5713 ± 2035</td>
<td>2625 ± 1753s</td>
</tr>
<tr>
<td>IL-10</td>
<td>bd</td>
<td>bd</td>
<td>bd</td>
<td>bd</td>
</tr>
<tr>
<td>KC</td>
<td>12355 ± 1829</td>
<td>8213 ± 1038</td>
<td>13561 ± 2698</td>
<td>11673 ± 1971 **</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1501 ± 1047</td>
<td>901 ± 299</td>
<td>1006 ± 265</td>
<td>1488 ± 1157</td>
</tr>
<tr>
<td>MIP-2</td>
<td>6329 ± 1802</td>
<td>2327 ± 720</td>
<td>9435 ± 2262</td>
<td>10359 ± 2590</td>
</tr>
</tbody>
</table>

Mice chimeric for TRIF were infected with $7 \times 10^3$ CFU K. pneumoniae 6 weeks after bone marrow transplantation. Forty-eight hours after infection, mice were sacrificed, lungs were removed and cytokine levels determined in lung homogenates. KC and MIP-2 were determined using ELISA. TNF-α, IL-6, IL-10 and MCP-1 levels were determined by Cytometric Bead Assay. Data are presented in pg/ml lung homogenate as mean ± SEM. N=9-11 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs R+/H+ levels determined with Mann Whitney U test. R recipient and H hematopoietic.