Host defense during *Klebsiella* pneumonia relies on hematopoietic expressed TLR4 and TLR2

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ABSTRACT

With this study the relative roles of Toll-like receptor (TLR)2 and -4 were investigated independently and together. Moreover, we studied the role of hematopoietic compartment in anti-Klebsiella host defense.

We infected TLR2, TLR4 and TLR2x4 double KO animals with different doses of K. pneumoniae. In addition, bone marrow chimeric mice were created and infected.

TLR4 played a more prominent role in antibacterial defense than TLR2, considering that only TLR4 KO mice demonstrated enhanced bacterial growth in lungs and spleen 24 h after infection with 3x10^3 Klebsiella compared to wild-type (WT) mice. In late stage infection or after exposure to a higher infectious dose, bacterial counts in lungs of TLR2 KO animals were elevated compared to WT mice, and TLR2x4 KO animals were more susceptible to infection than TLR4 KO mice. TLR signaling on cells from hematopoietic origin is of primary importance in host defense against K. pneumoniae.

These data suggest that (1) TLR4 drives the antibacterial host response after induction of pneumonia with relatively low Klebsiella doses; (2) TLR2 becomes involved at a later phase of the infection and/or upon exposure to higher bacterial burdens and (3) hematopoietic TLR2/4 are important for an adequate host response during Klebsiella pneumonia.

Key words: bacterial pneumonia, innate immunity, rodent, Toll-like receptors
INTRODUCTION

Gram-negative pneumonia is a common and serious illness that is a major cause of morbidity and mortality in humans. *Klebsiella (K.) pneumoniae* is a frequently isolated causative pathogen in nosocomial lower respiratory tract infection (1;2). The increasing microbial resistance to antibiotics, resulting in therapy failure and higher mortality rates, is an issue of major concern (1). Therefore, it is important to gain more insight into the pathogenesis of pneumonia.

Toll-like receptors (TLRs) recognize pathogens, resulting in onset of the inflammatory response (3). TLRs are expressed in both cells of hematopoietic origin and stromal cells (e.g. lung epithelium). When *K. pneumoniae* enters the lung, bacterium-specific TLRs are activated, triggering the release of cytokines and chemokines that attract and activate neutrophils. In the best case scenario, these neutrophils kill all bacteria after ingestion. TLR4 has been implicated as the most important TLR for the recognition of *K. pneumoniae* by virtue of its capacity to sense LPS present in the outer membrane of this Gram-negative pathogen (3). Indeed, in previous research we found that TLR4 mutant mice were highly susceptible to pulmonary infection with *K. pneumoniae* regardless of the infectious dose (4). The indispensable role of TLR4 for antibacterial defense against *Klebsiella* has subsequently been confirmed by other studies using different serotypes and different infection models (5-7). Notably, evidence indicates that other TLRs also contribute to host defense against *Klebsiella* pneumonia. Mice deficient for TLR9 (expressed within endosomes, recognizes
bacterial DNA (3)) had an impaired host defense after infection with *K. pneumoniae* via their airways, due to reduced dendritic cell accumulation and dendritic cell and macrophage activation in their lungs (8). Moreover, mice deficient for MyD88 (which mediates signaling of all TLRs except TLR3) or TIRAP (Toll-IL-1R domain-containing adaptor protein; an essential adapter for TLR1, TLR2, TLR4, and TLR6 signaling) displayed a diminished antibacterial defense during *Klebsiella pneumonia* (9;10).

In the present study we tested the hypothesis that TLR2, in conjunction with TLR4, is an important player in the protective immune response during respiratory tract infection by *K. pneumoniae*. In theory, TLR2 can contribute to the recognition of *Klebsiella* through an interaction with bacterial lipoproteins (3). Moreover, macrophages and dendritic cells can be activated by the *K. pneumoniae* pathogen associated molecular pattern (PAMP) outer membrane protein A (OmpA) through TLR2 (11). In addition, a recent study demonstrated that both TLR4 and TLR2 mRNA and protein are upregulated after stimulation of A549 cells (human lung epithelial cells) with *K. pneumoniae* (12). This study suggested that capsular polysaccharides are *Klebsiella* PAMPs responsible for TLR upregulation in lung epithelium.

We here infected mice deficient for TLR2, TLR4 or both TLR2 and TLR4 via the airways with *K. pneumoniae* and studied antibacterial host defense and immune responses. Interestingly, TLR2x4 double knockout (KO) mice were found to be more susceptible to *Klebsiella* pneumonia than animals deficient for TLR4 only. This study shows that TLR2 helps antibacterial host defense at a late stage of
the infection and/or upon exposure of the host to high bacterial numbers. Moreover, after creating bone marrow chimeric mice, we found that TLR2 and TLR4 expressed within the hematopoietic compartment are crucial for host defense against this nosocomial pathogen.
Materials and Methods

Animals

TLR4 (13) and TLR2 KO mice (14) were generously provided by Dr. S. Akira (Research Institute for Microbial Disease, Osaka, Japan). TLR2x4 KO mice were generated by intercrossing TLR2 KO and TLR4 KO mice. All genetically modified mice were back-crossed at least 6 times on a C57Bl/6 genetic background and bred in the animal facility of the Academic Medical Center, Amsterdam. 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

Pneumonia was induced as described previously (15). Briefly, *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA) was grown for 3 h to mid-logarithmic phase at 37°C using Tryptic Soy broth (Difco, Detroit, MI). Bacteria were harvested by centrifugation at 1500xg for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of 3x10^3 or 1x10^4 CFUs/50µl, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and bacteria were inoculated intranasally.
Determination of bacterial outgrowth

Five, 24 or 48 h after infection, mice were anesthetized with Domitor (Pfizer Animal Health Care, Capelle aan der IJssel, The Netherlands: active ingredient medetomidine) and Nimatek (Eurovet Animal Health, Bladel, The Netherlands, active ingredient ketamine) and sacrificed by heart puncture. Blood was collected in EDTA containing tubes. Lungs, liver and spleen were harvested and homogenized in sterile saline (1:5, weight/vol) using a tissue homogenizer (Biospec Products, Bartlesville, Oklahoma). CFUs in organ homogenates and blood were determined from serial dilutions plated on blood agar plates, incubated at 37°C for 16 h before colonies were counted.

Preparation of lung homogenates for cytokine measurements

For cytokine measurements, lungs were excised, weighed and homogenized in saline (1:5, weight/vol). Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 2 % Triton X-100, and AEBSF (4-(2-aminoethyl)benzeensulfonyl fluoride, EDTA-Na₂, pepstatin and leupeptin (all 8 μg/ml; pH 7.4) and incubated on ice for 30 min. Homogenates were centrifuged at 1500 × g at 4 °C for 15 min and stored at −20 °C until assays were performed. TNF, IL-1β, IL-6, keratinocyte-derived chemokine (KC) and MIP-2 were measured by ELISA using matched antibody pairs according to the manufacturer's instructions (R&D Systems Inc.,
Minneapolis, MN, USA). Detection limits were 63 pg/mL for TNF, IL-1β, IL-6 and MIP-2, and 15 pg/mL for KC.

**Histologic examination**

Lungs were removed and fixed in 10% formalin in PBS for 24 h and embedded in paraffin. Hematoxilin and eosin stained slides were coded and semi-quantitatively scored for inflammatory parameters by a pathologist who was not aware of the origin of the tissue samples. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, edema, endothelialitis, bronchitis and pleuritis. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: severe and 4: very severe. The percentage pneumonia was scored and graded according on a scale of 0 to 4, with 0: absent, 1: 5-20% confluent pneumonia, 2: 21-40%, 3: 41-60%, 4: 61-80% and 5: 81-100%. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 25 (15;16). Granulocyte staining was performed as described (17). In brief, slides were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by a solution of 0.1% NaN3/0.03% H2O2 (Merck). Slides were then digested by a solution of pepsin 0.25% (Sigma, St. Louis, MO) in 0.01 M HCl. After being rinsed, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labeled anti-mouse Ly-6-G monoclonal antibody (Pharmingen, San Diego, CA). After washes, slides were incubated with a rabbit anti-FITC
antibody (Dako) followed by further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidine-ABC solution (Dako) and developed using 1% H₂O₂ and 3.3-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were counterstained with methyl green and mounted in glycerin gelatin. The numbers of Ly-6G-positive cells were counted in 10 non-overlapping fields (×400)(18).

Bone marrow transplantation

To examine the relative roles of TLR2 plus TLR4 expression in hematopoietic (H) and structural (S) cells in the response to K. pneumoniae, we created bone marrow chimeric mice in essence as described previously (19; 20). Briefly, bone marrow cells were harvested from 7-9 week old WT (CD45.1⁺ or CD45.2⁺) and TLR2x4 KO (CD45.2⁺) mice (all age- and sexmatched). Cells were isolated by flushing tibia and femurs with PBS containing 10% FCS (BioWitthaker, Heidelberg, Germany), 100 U/ml penicillin (BioWitthaker), and 100 μg/ml streptomycin (BioWitthaker), and single cells were prepared by pulling the tissue clumps three times through a 25-gauge needle. Next, the cells were centrifuged at 250 x g for 10 minutes, aspirated, washed, and resuspended in PBS. At the start of the experiment recipient mice were six weeks old. The 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 5x10⁶ bone marrow cells from donor animals. To protect the irradiated recipient mice
from immediate infections, the mice were also injected with $2 \times 10^5$ 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 five weeks after transplantation, and they were housed in sterile filter top cages in a laminar flow chamber. Mice entered the infection experiment six weeks after bone marrow transplantation, one week after stopping the antibiotics. Engraftment was confirmed by flow cytometry in the peripheral blood just before starting the infection experiment. As a control for the transplantation procedure, we not only administered TLR2x4 KO bone marrow cells (H-) into WT recipient mice (S+) and WT bone marrow cells (H+) into TLR2x4 KO recipient mice (S-), but also WT bone marrow (H+) to WT mice (S+) and TLR2x4 KO bone marrow (H-) to TLR2x4 KO mice (S-). Thus, four groups of mice were generated (H+/S-, H-/S+ and as controls H+/S+ and H-/S-).

*Flow cytometry*

Blood was drawn by heart puncture and erythrocytes were lysed with ice-cold isotonic NH$_4$Cl solution (155 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA, pH 7.4); the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium), and counted by using a hemocytometer. The percentages of monocytes and neutrophils were determined using a FACSCalibur (BD, San Jose, CA). Cells were brought to a concentration of $1 \times 10^7$ cells/ml in FACs buffer (PBS supplemented with 0.5% PBS, 0.01% NaN$_3$ and 0.35 mM EDTA).
Immunostaining for cell surface molecules was performed for 30 minutes at 4°C using directly labeled antibodies (abs) against GR-1 (GR-1 FITC, BD Pharmingen, San Diego, California), CD45.1-phycoerythrin (CD45.1- PE; BD Pharmingen), CD45.2 Peridinin-chlorophyll-protein complex (CD45.2-PerCP, BD Pharmingen) and a allophycocyanine labeled antibody against F4/80 (Serotec, Oxford, United Kingdom). All abs were used in concentrations recommended by the manufacturer. Neutrophils were counted using the scatter pattern and GR-1 high gate, monocytes in the sidescatter low and F4/80 positive gate.

**Statistical analysis**

Data are expressed as mean ± SEM. Survival curves were compared using long rank test. Comparisons between multiple groups were performed using Kruskall-Wallis test with Mann Whitney U test as a post test using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). $P < 0.05$ was considered to be statistically significant.
Results

Delayed early inflammatory response in the absence of TLR4, but enhanced response in TLR2 KO mice

The success of combating pulmonary infections strongly depends on the efficacy of the local inflammatory response elicited. Early recognition by TLRs with subsequent release of chemokines (attraction immune cells) and cytokines (activation) has been proven to be crucial for successful host defense (21). We therefore studied bacterial growth, influx of neutrophils and levels of cytokines and chemokines after 5h of infection with $3 \times 10^3$ Klebsiella CFU. At this time point pulmonary bacterial loads were equal in the 4 mouse strains studied (fig. 1A), whereas cultures from distant organs remained sterile. In order to study the influx of neutrophils at this early time point, we performed immunohistochemical staining of lung tissue slides and counted the number of Ly6+ neutrophils in 10 high power fields (400x magnification). Despite no differences in pulmonary bacterial loads between WT, TLR2 KO, TLR4 KO and TLR2x4 KO mice, we found more neutrophils in lungs of TLR2 KO animals when compared to the other mouse strains (fig 1 B-D).

To further dissect the early inflammatory response, we measured several important cytokines and chemokines in lung homogenates (fig. 1E-H). When compared to WT mice, TLR2x4 KO mice demonstrated reduced lung levels of the chemokines KC and MIP-2 as well as the cytokines IL-1$\beta$ and IL-6. In TLR4 KO mice KC and IL-1$\beta$ were reduced 5h post infection. Interestingly, lungs of TLR2 KO animals contained significantly more IL-1$\beta$; moreover, KC and IL-6
demonstrated a trend towards higher levels in this mouse strain. No differences in TNF levels were detected in lung homogenates at this early time point of infection (data not shown).

**TLR2x4 double KO mice display a more profoundly disturbed antibacterial defense than TLR4 KO mice**

As a next step, we studied antibacterial host defense after 24h of infection, using two different bacterial doses: $3 \times 10^3$ CFU and $1 \times 10^4$ CFU. After inoculation with the lower bacterial dose, TLR4 and TLR2x4 double KO mice but not TLR2 KO mice had higher bacterial burdens in lung (fig. 2A) and spleen (fig. 2B) at this time point of infection. In the liver, only the mice that lacked both TLR2 and TLR4 had higher bacterial loads (fig. 2C). After infection with the higher inoculum, no differences were found in lung, spleen and liver of TLR2 and TLR4 KO mice at 24h (fig. 2D-E). Interestingly, TLR2x4 double KO mice had higher bacterial burdens in all organs examined (fig. 2D-E). These data suggest that antibacterial defense after low dose infection primarily is driven by TLR4, with a modest additional role for TLR2; however, after infection with a higher bacterial inoculum, apparently TLR2 can at least in part compensate for TLR4, considering that only TLR2x4 double KO mice, but not TLR4 KO mice, displayed enhanced bacterial outgrowth.

We did not find consistent differences between mouse strains in pulmonary cytokine and chemokine levels (Table I). After infection with $3 \times 10^3$ CFU TLR2x4 double KO mice displayed the lowest cytokine/chemokine levels, but the
differences with WT mice only were significant for IL-1β and TNF. TLR2 KO mice overall had similar mediator levels in their lungs as compared with WT mice, with the exception of IL-1β and MIP-2 which were higher in the former mouse strain after infection with the lower and higher bacterial inoculums respectively. Histopathology scores were in line with cytokine and chemokine levels: only in the lower dose infection, TLR2x4 displayed significantly reduced histopathology scores.

TLR2 is important during late stage of infection with *Klebsiella pneumonia*

Next we wished to study the late host response during *Klebsiella pneumonia*. For this we infected mice with $3 \times 10^3$ CFU, seeking to examine bacterial numbers and inflammatory responses 48h post infection. However, in the first experiment, in which we compared TLR2x4 double KO with WT mice, the former mouse strain proved to be hypersusceptible: all TLR2x4 double KO mice died between 24 and 48h after infection, whereas the first deaths amongst WT mice occurred beyond the 48h time point (fig. 3A). In a next experiment we infected WT, TLR2 KO and TLR4 KO mice with the same bacterial dose, again seeking to determine bacterial loads 48h post infection. Like TLR2x4 double KO mice, TLR4 KO mice demonstrated lethality beyond the 24h time point: whereas at 48h after infection all WT and TLR2 KO mice were alive, 5/8 TLR4 KO mice had died; the remaining 3 TLR4 KO mice showed high bacterial loads in all organs examined, especially in spleen and liver (fig. 3B-D). Most interestingly, bacterial numbers in lungs of TLR2 KO mice were higher than bacterial counts in lungs of WT mice. These
data again suggest that although TLR4 is pivotal for adequate host defense against *K. pneumoniae*, TLR2 plays a role in local antibacterial host defense in a late stage of the infection.

*TLR2x4 on hematopoietic cells are pivotal for host defense against* *Klebsiella*

TLRs are expressed on cells of hematopoietic origin and non-hematopoietic origin and both are potentially important in host defense against *Klebsiella*. To address whether TLR2x4 expression in either (radiosensitive) hematopoietic or (radioresistant) non-hematopoietic cells is sufficient for the innate immune response to *Klebsiella*, we generated bone marrow chimeric mice using WT and (the most susceptible strain) TLR2x4 double KO mice. In brief, either WT (CD45.1+) or TLR2x4 double KO mice (CD45.2+) were lethally irradiated and reconstituted with bone marrow from TLR2x4 double KO (CD45.2+) or WT mice (CD45.1+) respectively, creating WT mice reconstituted with TLR2x4 KO bone marrow (H-/S+) and TLR2x4 KO mice reconstituted with WT bone marrow (H+/S-). Control groups were also generated by transferring bone marrow from WT to WT mice (H+/S+) and from TLR2x4 KO to TLR2x4 KO mice (H-/S-). Engraftment was confirmed by flow cytometry in peripheral blood directly prior to induction of pneumonia (6 weeks after transplantation) and 24h after infection, revealing that, in accordance with our earlier data (19;20), the mean percentage of neutrophils and monocytes in blood of uninfected chimeras derived from the donor mouse was >90% in all groups transplanted; 24h after infection, the percentages of
donor neutrophils and monocytes in blood had remained > 90% in all groups (data not shown).

Six weeks after transplantation, we infected all groups with $10^4$ CFU *K. pneumoniae* and studied bacterial growth in lungs and spleen 24h later. The procedure of irradiation and bone marrow transfer did not affect host defense because the difference observed between irradiated WT mice reconstituted with WT bone marrow (H+/S+) and irradiated TLR2x4 KO mice reconstituted with TLR2x4 KO bone marrow (H-/S-) confirmed our earlier findings in non-irradiated animals: H-/S- mice displayed higher bacterial loads than H+/S+ mice in both lungs and spleen (fig. 4). Our main finding was that TLR2x4 KO mice reconstituted with WT bone marrow (H+/S-) displayed equal amounts of bacteria in lungs and spleen when compared to H+/S+ animals. Moreover, irradiated WT reconstituted with TLR2x4 KO bone marrow were more susceptible to infection: H-/S+ mice displayed increased bacterial outgrowth in lung and spleen when compared to H+/S+ mice. Finally, bacterial loads in H-/S+ and H-/S- mice were similar. Together, these findings demonstrate the importance of TLR2/4 expression on radiosensitive hematopoietic cells for an adequate antibacterial defense during *Klebsiella pneumonia*.

In line with earlier experiments (table 1), pulmonary levels of KC and IL-1β were reduced in H-/S- mice in comparison to H+/S+ mice despite higher bacterial loads (table 3). Mice with TLR2/4 deficient stroma (S-) demonstrated reduced KC levels regardless of the origin of reconstituted hematopoietic cells (H+ or H-), pointing to KC being mainly produced by stromal cells like epithelia.
Discussion

*K. pneumoniae* is a clinically important Gram-negative pathogen in hospital-acquired pneumonia. Previous research demonstrated that TLR4 is important for an adequate host defense in *K. pneumoniae* pneumonia (4;5;7). Considering that *Klebsiella* expresses several TLR2 ligands (3;11), we here sought to determine the role of TLR2, in the presence or absence of functional TLR4, in the innate immune response to respiratory tract infection by this bacterium in vivo. In addition, we aimed to assess the relative contribution of TLR2/4 on hematopoietic and stromal cells herein. Our main findings were: (1) TLR4 drives the antibacterial host response after infection with relatively low *Klebsiella* doses, (2) TLR2 becomes involved at a later phase of the infection and/or upon exposure of the host to higher bacterial burdens and (3) TLR2/4 expressed by radiosensitive hematopoietic cells and not by radioresistant stromal cells are important for an adequate host response.

*Klebsiella* pneumonia and pneumosepsis are common in and outside the hospital environment (1;2). Nosocomial pneumonia mainly affects patients with pre-existing diseases that may impact on host defense in the lung. Our model uses previously healthy mice and such more resembles community-acquired *Klebsiella* pneumonia. Other laboratories have used the same method to obtain insight into the innate immune response during respiratory tract infection by this pathogen (22). Of note, the ATCC strain of *K. pneumoniae* serotype 2 used in this study is a common laboratory strain that was used in many studies.
investigating host response against *K. pneumoniae*. The capsular serotypes 1 and 2 are the most common and the most virulent *Klebsiella* serotypes (2;23;24). Upon infection of TLR2 KO mice with *K. pneumoniae*, we discovered a dual role for TLR2. In the early initial recognition phase, TLR2-related pathways delayed IL-1β release and neutrophil influx. We previously observed a similar dampening function of TLR2 in the early host response during another Gram-negative pneumonia, caused by *Acinetobacter baumannii* (25). Along the same line, TLR2 KO mice were reported to be less susceptible to lethal infections with *Yersinia enterocolitica* or *Candida albicans* through a mechanism that involved a stronger type 1 cytokine response (26;27). Although the exact mechanisms behind these possible anti-inflammatory properties of TLR2 remain unclear, it is possible that lack of TLR2 signaling was associated with upregulation of other receptors with mainly pro-inflammatory properties, such as has been described for TLR4 in TLR2 KO mice infected with *Pseudomonas aeruginosa* (28). Interestingly, the role of TLR2 changed during the course of infection. While TLR2 deficiency did not impact on bacterial growth early after infection with a low bacterial dose, at a later stage or after infection with a higher inoculum, TLR2 did contribute to antibacterial defense. Indeed, relative to WT mice, TLR2 KO mice had higher bacterial counts in lungs 48 (but not 24) h after infection with $3 \times 10^3$ *Klebsiella* CFU, whereas TLR2x4 double KO mice displayed higher bacterial burdens than TLR4 KO mice in lungs and distant organs 24 h after infection with $10^4$ *Klebsiella* CFU, which was associated with an accelerated lethality beyond this time point. TLR4 clearly played a more prominent role in antibacterial defense than TLR2,
especially in the initial phase of the infection, considering that TLR4 KO (but not TLR2 KO) mice demonstrated enhanced bacterial growth in lungs and spleen 24 h after infection with $3 \times 10^3$ *Klebsiella* CFU when compared with WT mice and considering that TLR4 KO mice and TLR2x4 double KO mice harbored equal bacterial loads in lungs and spleen at this time point. Together, these data suggest that the interaction between LPS and TLR4 drives the early host response during *Klebsiella* pneumonia, whereas the interaction between TLR2 ligands expressed by this bacterium and TLR2 becomes a factor upon exposure to higher bacterial numbers.

In clinical practice, patients would have been treated with antibiotics before lethality occurred. It is very well possible that the outcome in the TLR deficient animals would be different in the context of antibiotic therapy. Hypothetically, it might be beneficial to lack TLR mediated hyperinflammation when antibiotics are taking care of bacterial elimination. It is therefore important to realize that the primary aim of our study was to determine the role of TLRs in the innate immune response during *Klebsiella* pneumonia, rather than to investigate the therapeutic potential of TLR inhibition (which clearly should be studied in animals concurrently treated with antibiotics).

Neutrophil recruitment to the lungs is an important first line of defense against bacterial infections (21). However, in addition to neutrophils and lung macrophages, non hematopoietic cells, such as lung epithelium and endothelium, contribute to the initial recognition of bacteria and production of inflammatory mediators and thus host defense. To dissect the role of
hematopoietic cells and cells of nonhematopoietic origin, we generated bone marrow chimeric mice using WT and TLR2x4 double KO mice, the most susceptible mouse strain. We found that injecting WT bone marrow into irradiated TLR2x4 KO animals resulted in similar bacterial growth as in syngenic transplanted WT mice and reduced outgrowth when compared to syngenic TLR2x4 KO animals, meaning that hematopoietic cells are of utmost importance in host defense. In line, irradiated WT mice that received TLR2x4 KO bone marrow did worse than syngenic transplanted WT animals. Since differences in antibacterial host defense between syngenic transplanted (H+/S+ and H-/S-) mice on the one hand and non-transplanted WT and TLR2x4 KO animals on the other hand were similar, we believe that our results are not an artifact introduced by the bone marrow transplantation procedure.

Several earlier studies investigated the role of TLRs in host defense against Klebsiella pneumonia. In accordance with the current data, C3H/HeJ mice (which harbor a mutation in TLR4 that renders this receptor dysfunctional) were reported to have an enhanced bacterial growth and dissemination and a reduced survival (4;5). TLR4 can signal via two intracellular routes, relying on the adaptors TRIF (TIR domain-containing adaptor-inducing IFN-β) and MyD88 respectively (3), and both TRIF KO and MyD88 KO displayed an impaired host defense during Klebsiella pneumonia (9). Notably, MyD88 mediates signaling of all TLRs excluding TLR3 and of the signaling receptors for IL-1 and IL-18 (29); the possibility that multiple TLRs are involved in protective immunity during
respiratory tract infection by *K. pneumoniae* is further supported by investigations revealing an enhanced susceptibility of mice deficient for either TLR9 (8) or TIRAP (which mediates signaling of TLR1, TLR2, TLR4, and TLR6)(10). We here expand these previous data showing that during pneumonia caused by *K. pneumoniae* TLR4 and TLR2 expressed by hematopoietic cells interact in mediating an effective antibacterial defense in a manner that is dependent on the stage of the infection and the bacterial load to which the host is exposed. The present study further suggests that TLR2 plays a dual role in the host response to *Klebsiella pneumonia*: while TLR2 signaling dampens the initial inflammatory response after relatively low dose infection without influencing bacterial expansion, at later stages TLR2 is important in limiting bacterial growth irrespective of the presence of TLR4.
Acknowledgements

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Reference List


Table 1:

Lung cytokine/chemokine levels and histopathology scores 24h after infection

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</tbody>
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*aWT, TLR2, TLR4 and TLR2x4 KO mice were infected with the indicated inoculum of K. pneumoniae. After 24h, mice were sacrificed, right lungs were removed and KC, MIP-2, IL-1β and IL-6 were determined using ELISA (pg/mL). Left lungs were used for determining histopathology scores as described in the Methods section. Data are means ± SEM, n = 7 or 8 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs WT levels.
Table 2:
Lung cytokine and chemokine levels in chimeric mice 24h after infection\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>KC</th>
<th>MIP-2</th>
<th>IL-1\textbeta</th>
<th>IL-6</th>
<th>TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>H+/S+</td>
<td>11224 ± 778</td>
<td>22822 ± 11200</td>
<td>1949 ± 472</td>
<td>859 ± 156</td>
<td>1368 ± 302</td>
</tr>
<tr>
<td>H+/S-</td>
<td>6115 ± 1605*</td>
<td>32113 ± 12403</td>
<td>3411 ± 1122</td>
<td>1271 ± 236</td>
<td>1944 ± 648</td>
</tr>
<tr>
<td>H-/S+</td>
<td>11198 ± 682</td>
<td>34712 ± 11590</td>
<td>2674 ± 490</td>
<td>1328 ± 176</td>
<td>1755 ± 395</td>
</tr>
<tr>
<td>H-/S-</td>
<td>6954 ± 1321*</td>
<td>4245 ± 1035</td>
<td>855 ± 154*</td>
<td>1106 ± 129</td>
<td>1978 ± 425</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Chimeric mice were infected with 4x10\textsuperscript{4} CFU \textit{K. pneumoniae} 6 weeks after bone marrow transplantation. Twenty-four h later, mice were sacrificed, lungs were removed and KC, MIP-2, IL-1\textbeta and IL-6 were determined using ELISA (pg/mL). Data are means ± SEM, n = 7 or 8 mice per group. *\textit{P}<0.05, **\textit{P}<0.01, ***\textit{P}<0.001 vs WT levels. H hematopoietic ; S structural
Figure legends

Fig. 1: Impact of TLR4 and TLR2 on the early inflammatory response during Klebsiella pneumonia

WT, TLR2, TLR4 and TLR2x4 KO mice were inoculated with K. pneumoniae (3x10³ CFU) and lung bacterial counts were determined after 5 h of infection (A). Each symbol represents an individual mouse. Horizontal lines indicate medians. The number of Ly6+ neutrophils (B, t = 5h) was significantly higher in TLR2 KO mice (representative picture shown in panel D, original magnification x 40) compared to WT animals (representative picture shown in panel C) after Klebsiella pneumonia as counted in 10 randomly selected high-power fields (B). Chemokines KC (E) and MIP-2 (F) and cytokines IL-1β (G) and IL-6 (H) were measured in lung homogenates. Data were shown as means ± SEM of 8 mice per group. *P<0.05; **P<0.01, ***P<0.001 vs WT mice.
Fig. 2: TLR4 KO mice demonstrate enhanced bacterial outgrowth after low dose infection, while after high dose infection bacterial loads are only increased in TLR2x4 double KO mice

WT, TLR2, TLR4 and TLR2x4 KO mice were inoculated with two doses K. pneumoniae: 3x10^3 CFU (A-C) or 1x10^4 CFU (D-F). After 24 h of infection, bacterial burdens were determined in lung (A, D), spleen (B, E) and liver (C, F) homogenates. Each symbol represents an individual mouse. Horizontal lines indicate medians. *P<0.05; **P<0.01 vs WT mice; # P<0.05 vs TLR4 KO mice.
Fig. 3: TLR2 is important during the late phase of infection

(A) WT (n = 8; closed symbols) and TLR2x4 KO mice (n=8; open symbols) were inoculated with 3x10^3 CFU *K. pneumoniae* and followed for 14 days. *P* < 0.001.
(B) WT, TLR2 and TLR4 mice (n=8 mice per group) were inoculated with 3x10^3 *K. pneumoniae*. After 48 h of infection, bacterial burdens were determined in lung (B), spleen (C) and liver (D) homogenates of the remaining animals. Each symbol represents an individual mouse. Horizontal lines indicate medians. *P*<0.05 vs WT mice.

Fig. 4: TLR2/4 expressed by hemapoietic cells are important for antibacterial defense during *K. pneumoniae* pneumonia

WT (S+) and TLR2x4 KO (S-) mice were irradiated and injected with WT (H+) or TLR2x4 KO (H-) bone marrow cells. Six weeks after transplantation, mice were infected with 10^4 CFU *K. pneumoniae* and sacrificed 24h later. Pulmonary (A) and splenic (B) outgrowth of *K. pneumoniae* was determined in organ
homogenates. Each symbol represents an individual mouse. Horizontal lines indicate medians. *$P<0.05$; **$P<0.01$ vs H+/S+. ns = not significant.