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Host defence during *Klebsiella* pneumonia relies on haematopoietic-expressed Toll-like receptors 4 and 2

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ABSTRACT: In this study, the relative roles of Toll-like receptor (TLR)2 and TLR4 were investigated independently and together. Moreover, we studied the role of haematopoietic compartment in anti-*Klebsiella* host defence.

We infected TLR2 and TLR4 single-, and TLR2 × 4 double knockout (KO) animals with different doses of *Klebsiella pneumoniae*. In addition, bone marrow chimeric mice were created and infected.

TLR4 played a more prominent role in antibacterial defence than TLR2, considering that only TLR4 KO mice demonstrated enhanced bacterial growth in lungs and spleen 24 h after infection with 3×10^3 colony-forming units of *Klebsiella* compared with 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 with WT mice and TLR2 × 4 KO animals were more susceptible to infection than TLR4 KO mice. TLR signalling in cells of haematopoietic origin is of primary importance in host defence 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) haematopoietic TLR2 and TLR4 are important for an adequate host response during *Klebsiella* pneumonia.

KEYWORDS: Bacterial pneumonia, innate immunity, rodent, Toll-like receptors

Pneumonia caused by Gram-negative bacteria is a common and serious illness that is a major cause of morbidity and mortality in humans. *Klebsiella 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) recognise pathogens, resulting in onset of the inflammatory response [3]. TLRs are expressed in both cells of haematopoietic 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 lipolysaccharide (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 defence against *Klebsiella* has subsequently been confirmed by other studies using different serotypes and infection models [5–7]. Notably, evidence indicates that other TLRs also contribute to host defence against *Klebsiella* pneumonia. Mice deficient for TLR9 (which is expressed within endosomes and recognises bacterial DNA [3]) had an impaired host defence 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 (myeloid differentiation primary response gene 88; which mediates signalling of all TLRs except TLR3) or TIRAP (Toll–interleukin-1 receptor domain-containing adaptor protein; an essential adapter for TLR1, TLR2, TLR4 and TLR6 signalling) displayed a diminished antibacterial defence during *Klebsiella* pneumonia [9, 10].

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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]. That study suggested that capsular polysaccharides are *Klebsiella* PAMPs responsible for TLR upregulation in lung epithelium.

In the present study, we infected mice deficient for TLR2, TLR4 or both TLR2 and TLR4 *via* the airways with *K. pneumoniae*, and studied antibacterial host defence and immune responses. Interestingly, TLR2×4 double 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 defence 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 haematopoietic compartment are crucial for host defence against this nosocomial pathogen.

MATERIALS AND METHODS

Animals

TLR4 [13] and TLR2 KO mice [14] were generously provided by S. Akira (Research Institute for Microbial Disease, Osaka, Japan). TLR2×4 KO mice were generated by intercrossing TLR2 KO and TLR4 KO mice. All genetically modified mice were back-crossed at least six times on a C57Bl/6 genetic background and bred in the animal facility of the Academic Medical Center (University of Amsterdam, Amsterdam, the Netherlands). 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, USA) was grown for 3 h to mid-logarithmic phase at 37°C using tryptic soy broth (Difco, Detroit, MI, USA). Bacteria were harvested by centrifugation at 1,500x*g* for 15 min and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of 3×10^3 or 1×10^4 colony-forming units (CFU)·50 μL^{-1}, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anaesthetised by inhalation of isoflurane (Upjohn, Ede, the Netherlands) and bacteria were inoculated intranasally.

Determination of bacterial outgrowth

5, 24 or 48 h after infection, mice were anaesthetised with medetomidine (Domitor, Pfizer Animal Health Care, Capelle aan der IJssel, the Netherlands) and ketamine (Nimatek, Eurovet Animal Health, Bladel, the Netherlands), and sacrificed by heart puncture. Blood was collected in EDTA-containing tubes. Lungs, liver and spleen were harvested and homogenised in sterile saline (weight:volume 1:5) using a tissue homogeniser (Biospec Products, Bartlesville, OK, USA). Colony-forming units 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 homogenised in saline (weight:volume 1:5). Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM tris-(hydroxymethyl)-aminomethane (Tris), 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2 % Triton X-100 and 4-(2-aminoethyl)-benzenesulfonyl fluoride, Na_2EDTA, pepstatin and leupeptin (all 8 $\mu g \cdot mL^{-1}$, pH 7.4) and incubated on ice for 30 min. Homogenates were centrifuged at $1,500\times g$ at 4°C for 15 min and stored at -20°C until assays were performed. Tumour necrosis factor (TNF), interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (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 $pg \cdot mL^{-1}$ for TNF, IL-1β, IL-6 and MIP-2 and 15 $pg \cdot mL^{-1}$ for KC.

Histological examination

Lungs were removed and fixed in 10% formalin in PBS for 24 h and embedded in paraffin. Haematoxylin- and eosin-stained slides were coded and semiquantitatively 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 analysed with respect to the following parameters: interstitial inflammation, oedema, endothelialitis, bronchitis and pleuritis. Each parameter was graded on a scale of 0–4, (0: absent; 1: mild; 2: moderate; 3: severe; 4: very severe). The percentage pneumonia was scored and graded according on a scale of 0–4 (0: absent; 1: 5–20% confluent pneumonia; 2: 21–40%; 3: 41–60%; 4: 61–80%; 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 previously [17]. In brief, slides were deparaffinised and rehydrated. Endogenous peroxidase activity was quenched with a solution of 0.1% NaN_3 and 0.03% H_2O_2 (Merck, Whitehouse Station, NJ, USA). Slides were then digested with a solution of 0.25% pepsin (Sigma, St Louis, MO, USA) in 0.01 M HCl. After being rinsed, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to fluorescein isothiocyanate (FITC)-labelled anti-mouse Ly-6-G monoclonal antibody (Pharmingen, San Diego, CA, USA). After washes, slides were incubated with a rabbit anti-FITC antibody (Dako) followed by further incubation with a biotinylated pig anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidin–horseradish peroxidase solution (Dako) and developed using 1% H_2O_2 and 3,3-diaminobenzidine tetrahydrochoride (Sigma) in Tris–HCl. The sections were counterstained with methyl green and mounted in glycerine gelatine. The numbers of Ly-6G-positive cells were counted in 10 nonoverlapping fields (at 400× magnification) [18].

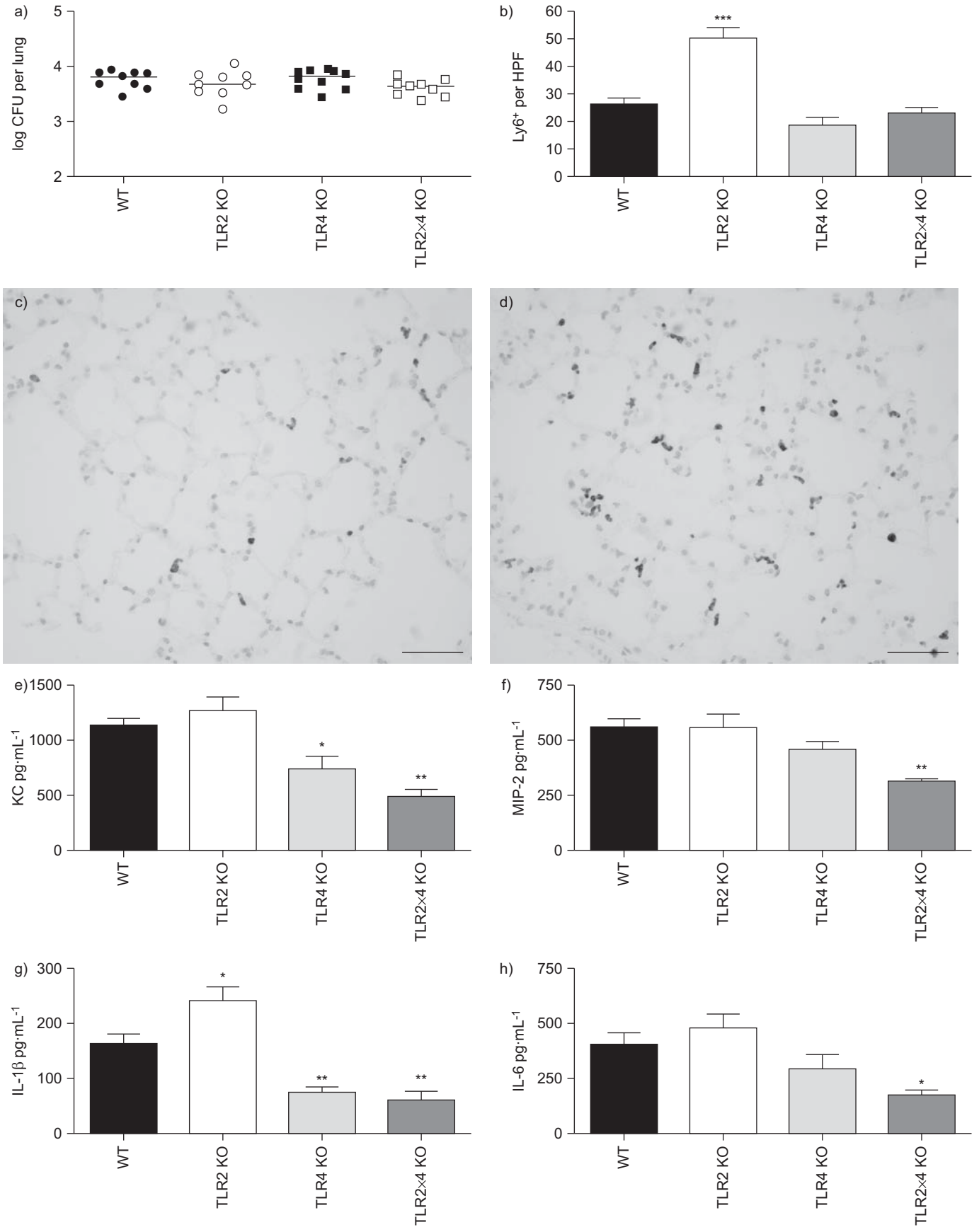
a)
log CFU per lung
5
4
3
2
WT
TLR2 KO
TLR4 KO
TLR2×4 KO
b)
Ly6+ per HPF
60
50
40
30
20
10
0
***
WT
TLR2 KO
TLR4 KO
TLR2×4 KO
c)
d)
e)
KC pg·mL-1
1500
1000
500
0
*
**
WT
TLR2 KO
TLR4 KO
TLR2×4 KO
f)
MIP-2 pg·mL-1
750
500
250
0
**
WT
TLR2 KO
TLR4 KO
TLR2×4 KO
g)
IL-1β pg·mL-1
300
200
100
0
*
**
**
WT
TLR2 KO
TLR4 KO
TLR2×4 KO
h)
IL-6 pg·mL-1
750
500
250
0
*
WT
TLR2 KO
TLR4 KO
TLR2×4 KO

FIGURE 1. Impact of Toll-like receptor (TLR)4 and TLR2 on the early inflammatory response during *Klebsiella* pneumonia. a) Wild-type (WT), TLR2, TLR4 and TLR2×4 knockout (KO) mice were inoculated with *Klebsiella pneumoniae* (3×10^3 colony-forming units (CFU)) and lung bacterial counts were determined after 5 h of infection. Each symbol represents an individual mouse. —: median. b) The number of Ly6+ neutrophils (t=5 h) was significantly higher in TLR2 KO mice (representative picture shown in d; scale bars=50 μm) compared with WT animals (representative picture shown in c) after *K. pneumonia* as counted in 10 randomly selected high-power fields (HPFs). Chemokines e) keratinocyte-derived chemokine (KC) and f) macrophage inflammatory protein (MIP)-2, and cytokines g) interleukin (IL)-1β and h) IL-6 were measured in lung homogenates. Data are presented as mean±SEM of 8 mice per group. *: $p<0.05$ *versus* WT; **: $p<0.01$ *versus* WT; ***: $p<0.001$ *versus* WT.

Bone marrow transplantation

To examine the relative roles of TLR2 and TLR4 expression in haematopoietic (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 TLR2×4 KO (CD45.2+) mice (all age- and sex-matched). Cells were isolated by flushing tibia and femurs with PBS containing 10% fetal calf serum (BioWitthaker, Heidelberg, Germany), 100 U·mL^{-1} penicillin (BioWitthaker) and 100 μg·mL^{-1} 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 min, aspirated, washed and resuspended in PBS. At the start of the experiment, recipient mice were 6 weeks old. The recipient groups received a lethal total body irradiation of 2×4.5 Gy with 3 h between the two doses, using a ^{137}Cs irradiator (CIS Bio International, Gif, France) at a dose rate of 0.5 Gy·min^{-1}, followed by intravenous injection of 5×10^6 bone marrow cells from donor animals. To protect the irradiated recipient mice from immediate infections, the mice were also injected with 2×10^5 splenocytes from donor animals that were crushed through a 40-μm filter, washed and resuspended in PBS. Moreover, mice were provided with autoclaved, acidified drinking water containing 0.16% neomycin sulfate (Sigma) from 1 week before until 5 weeks after transplantation, and they were housed in sterile filter-top cages in a laminar flow chamber. Mice entered the infection experiment 6 weeks after bone marrow transplantation (1 week after stopping the antibiotics). Engraftment was confirmed by flow cytometry of the peripheral blood just before starting the infection experiment. As a control for the transplantation procedure, we not only administered TLR2×4 KO bone marrow cells (H-) into WT recipient mice (S+) and WT bone marrow cells (H+) into TLR2×4 KO recipient mice (S-), but also WT bone marrow (H+) to WT mice (S+) and TLR2×4 KO bone marrow (H-) to TLR2×4 KO mice (S-). Thus, four groups of mice were generated (H+/S- and 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_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4); the remaining cells were washed twice with RPMI 1640 (Bio Whiitaker, Verviers, Belgium), and counted by using a haemocytometer. The percentages of monocytes and neutrophils were determined using a FACSCalibur (BD, San Jose, CA, USA). Cells were brought to a concentration of 1×10^7 cells·mL^{-1} in fluorescence-activated cell sorting buffer (PBS supplemented with 0.5% bovine serum albumin, 0.01% NaN_3 and 0.35 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using directly labelled antibodies (Abs) against GR-1 (GR-1 FITC; BD Pharmingen, San Diego, CA, USA), CD45.1 (CD45.1– phycoerythrin; BD Pharmingen), CD45.2 (CD45.2–peridinin-chorophyll protein complex; BD Pharmingen) and an allophycocyanine-labeled antibody against F4/80 (Serotec; Oxford, UK). All Abs were used in concentrations recommended by the manufacturer. Neutrophils were counted using the scatter pattern and GR-1 high gate; monocytes were counted in the sidescatter low and F4/80-positive gate.

Statistical analysis

Data are presented as mean±SEM. Survival curves were compared using the log-rank test. Comparisons between multiple groups were performed using the Kruskall–Wallis test with the Mann–Whitney U-test as a post-test, using GraphPad Prism version 4.00 (GraphPad Software; San Diego, CA, USA). p-values <0.05 were 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 combatting 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 defence [21]. We, therefore, studied bacterial growth, influx of neutrophils, and levels of cytokines and chemokines after 5 h of infection with 3×10^3 CFU *Klebsiella*. At this time-point pulmonary bacterial loads were equal in the four 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 (400× magnification). Despite no differences in pulmonary bacterial loads between WT, TLR2 KO, TLR4 KO and TLR2×4 KO mice, we found more neutrophils in lungs of TLR2 KO animals when compared to the other mouse strains (fig. 1b–d).

In order 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, TLR2×4 KO mice demonstrated reduced lung levels of the chemokines KC and MIP-2, and the cytokines IL-1β and IL-6. In TLR4 KO mice, KC and IL-1β were reduced 5 h after infection. Interestingly, lungs of TLR2 KO animals contained significantly more IL-1β; 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).

TLR2×4 double KO mice display a more profoundly disturbed antibacterial defence than TLR4 KO mice

As a next step, we studied antibacterial host defence after 24 h of infection, using two different bacterial doses: 3×10^3 and

1×10^4 CFU. After inoculation with the lower bacterial dose, TLR4 and TLR2 × 4 double KO mice but not TLR2 KO mice had higher bacterial burdens in lung (fig. 2a) and spleen (fig. 2c) at this time-point of infection. In the liver, only the mice that lacked both TLR2 and TLR4 had higher bacterial loads (fig. 2e). After infection with the higher inoculum, no differences were found in lung, spleen and liver of TLR2 and TLR4 KO mice at 24 h (fig. 2b and d). Interestingly, TLR2 × 4 double KO mice had higher bacterial burdens in all organs examined (fig. 2b and e). These data suggest that antibacterial defence after low-dose infection is primarily driven by TLR4, with a modest additional role for TLR2; however, after infection with a higher

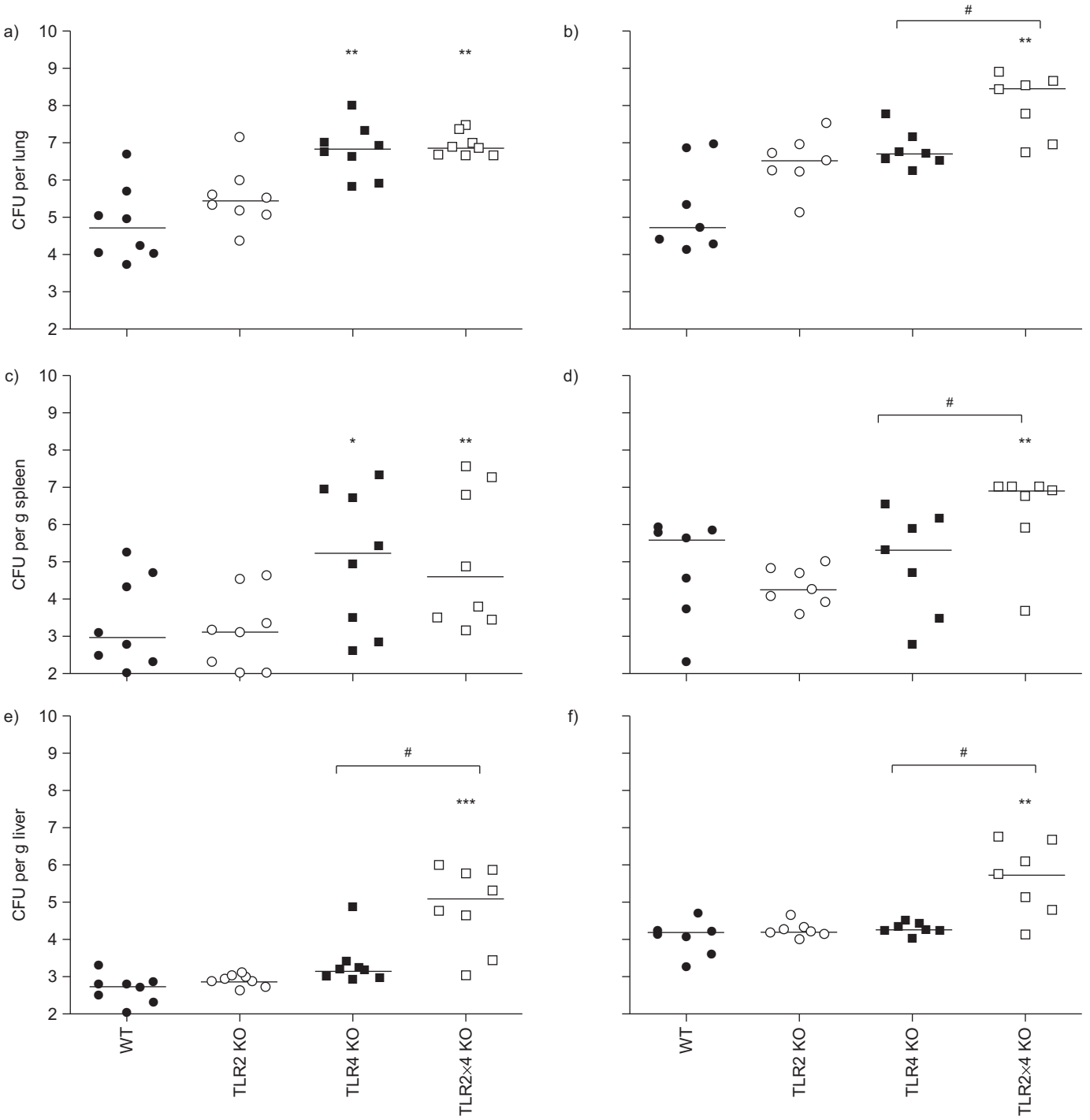

FIGURE 2. Toll-like receptor (TLR)4 knockout (KO) mice (■) demonstrate enhanced bacterial outgrowth after low-dose infection, while after high-dose infection, bacterial loads are only increased in TLR2 × 4 double KO mice (□). Wild-type (WT; ●), TLR2 (○), TLR4 and TLR2 × 4 KO mice were inoculated with two doses *Klebsiella pneumoniae*: a, c, e) 3×10^3 or b, d, f) 1×10^4 colony-forming units (CFU). After 24 h of infection, bacterial burdens were determined in a, b) lung, c, d) spleen and e, f) liver homogenates. Each symbol represents an individual mouse. —: median. *: $p<0.05$ *versus* WT; **: $p<0.01$ *versus* WT; #: $p<0.05$ *versus* TLR4 KO.

bacterial inoculum, TLR2 can apparently, at least partially, compensate for TLR4, considering that only TLR2×4 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 1). After infection with 3×10^3 CFU *K. pneumoniae*, TLR2×4 double KO mice displayed the lowest cytokine and chemokine levels, but the differences compared with WT mice only were significant for IL-1β and TNF. Overall, TLR2 KO mice had similar mediator levels in their lungs as compared to WT mice, with the exception of IL-1β and MIP-2, which were higher in TLR2 KO mice after infection with the lower and higher bacterial inoculums, respectively. Histopathology scores were consistent with cytokine and chemokine levels: only in the low-dose infection did TLR2×4 mice display significantly reduced histopathology scores.

TABLE 1 Lung cytokine and chemokine levels, and histopathology scores 24 h after infection

	Inoculum CFU	
	3×10^3	1×10^4
KC pg·mL^{-1}		
WT	4150±477	6360±667
TLR2 KO	3688±346	5490±432
TLR4 KO	3261±698	3982±1023
TLR2×4 KO	2035±471	4459±1056
IL-1β pg·mL^{-1}		
WT	2401±309	1936±391
TLR2 KO	3842±620*	2504±302
TLR4 KO	1549±436	799±301
TLR2×4 KO	781±580***	1763±580
TNF pg·mL^{-1}		
WT	188±18	965±103
TLR2 KO	256±29	1551±341
TLR4 KO	146±13*	650±130
TLR2×4 KO	129±2**	719±147
MIP-2		
WT	4290±1308	12782±3566
TLR2 KO	6132±848	32579±1671**
TLR4 KO	3137±862	6466±2831
TLR2×4 KO	1717±526	17088±5739
IL-6		
WT	1602±325	1528±480
TLR2 KO	2934±580	3355±776
TLR4 KO	1964±445	1637±667
TLR2×4 KO	1452±319	3693±1473
Histopathological score		
WT	7.3±0.6	10.1±1.2
TLR2 KO	8.5±0.6	10.5±0.4
TLR4 KO	6.2±0.9	7.9±1.0
TLR2×4 KO	4.6±0.7**	9.4±1.1

Data are presented as mean±SEM. Wild-type (WT), Toll-like receptor (TLR)2, TLR4 and TLR2×4 knockout (KO) mice were infected with the indicated inocula of *Klebsiella pneumoniae*. After 24 h, mice were sacrificed, right lungs were removed and keratinocyte-derived chemokine (KC), interleukin (IL)-1β, tumour necrosis factor (TNF), macrophage inflammatory protein (MIP)-2 and IL-6 were determined using ELISA. Left lungs were used for determining histopathology scores as described in the Methods section. n=7–8 mice per group. *: $p<0.05$ *versus* WT; **: $p<0.01$ *versus* WT; ***: $p<0.001$ *versus* WT.

TLR2 is important during late-stage infection with K. pneumoniae

Next, we wished to study the late host response during *Klebsiella* pneumonia. For this, we infected mice with 3×10^3 CFU *K. pneumoniae*, seeking to examine bacterial numbers and inflammatory responses 48 h after infection. However, in the first experiment, in which we compared TLR2×4 double KO with WT mice, the former mouse strain proved to be hypersusceptible: all TLR2×4 double KO mice died 24–48 h after infection, whereas the first deaths amongst WT mice occurred beyond the 48-h time-point (fig. 3a). In the next experiment, we infected WT, TLR2 KO and TLR4 KO mice with the same bacterial dose, again seeking to determine bacterial loads 48 h after infection. Like TLR2×4 double KO mice, TLR4 KO mice demonstrated lethality beyond the 24-h time-point. Whereas at 48 h after infection, all WT and TLR2 KO mice were alive, five out of eight TLR4 KO mice had died; the remaining three 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 defence against *K. pneumoniae*, TLR2 plays a role in local antibacterial host defence in a late stage of the infection.

TLR2 and TLR4 on haematopoietic cells are pivotal for host defence against K. pneumoniae

TLRs are expressed on cells of haematopoietic and nonhaematopoietic origin, and both are potentially important in host defence against *Klebsiella*. To address whether TLR2 and TLR4 expression in either (radiosensitive) haematopoietic or (radioresistant) nonhaematopoietic cells is sufficient for the innate immune response to *Klebsiella*, we generated bone marrow chimeric mice using WT and TLR2×4 double KO (the most susceptible strain) mice. In brief, either WT (CD45.1+) or TLR2×4 double KO (CD45.2+) mice were lethally irradiated and reconstituted with bone marrow from TLR2×4 double KO (CD45.2+) or WT mice respectively, creating WT mice reconstituted with TLR2×4 KO bone marrow (H-/S+) and TLR2×4 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 TLR2×4 KO to TLR2×4 KO mice (H-/S-). Engraftment was confirmed by flow cytometry on peripheral blood directly prior to induction of pneumonia (6 weeks after transplantation) and 24 h 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. 24 h after infection, the percentages of donor neutrophils and monocytes in blood had remained >90% in all groups (data not shown).

6 weeks after transplantation, we infected all groups with 10^4 CFU *K. pneumoniae* and studied bacterial growth in lungs

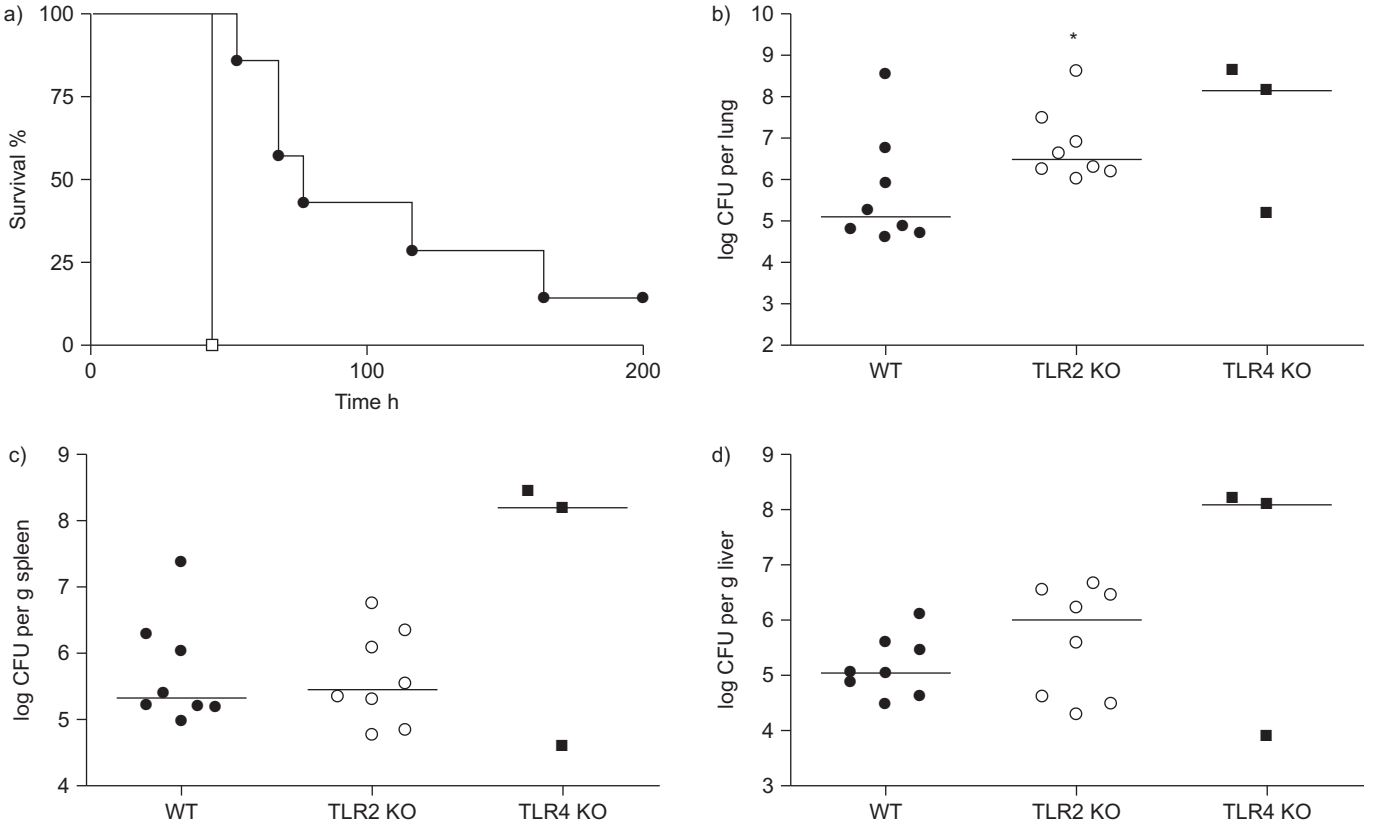

FIGURE 3. Toll-like receptor (TLR)2 is important during the late phase of infection. a) Wild-type (WT; n=8; ●) and TLR2×4 knockout (KO) mice (n=8; □) were inoculated with 3×10^3 colony-forming units (CFU) *Klebsiella pneumoniae* and followed for 14 days. $p<0.001$. b) WT, TLR2 KO (○) and TLR4 KO (■) mice (n=8 mice per group) were inoculated with 3×10^3 CFU *K. pneumoniae*. After 48 h of infection, bacterial burdens were determined in b) lung, c) spleen and d) liver homogenates of the remaining animals. Each symbol represents an individual mouse. —: median. *: $p<0.05$ *versus* WT.

and spleen 24 h later. The procedure of irradiation and bone marrow transfer did not affect host defence, because the difference observed between irradiated WT mice reconstituted with WT bone marrow (H+/S+) and irradiated TLR2×4 KO mice reconstituted with TLR2×4 KO bone marrow (H-/S-) confirmed our earlier findings in nonirradiated animals: H-/S- mice displayed higher bacterial loads than H+/S+ mice in both lungs and spleen (fig. 4). Our main finding was that TLR2×4 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 mice reconstituted with TLR2×4 KO bone marrow were more susceptible to infection: H-/S+ mice displayed increased bacterial outgrowth in lung and spleen compared with H+/S+ mice. Finally, bacterial loads in H-/S+ and H-/S- mice were similar. Together, these findings demonstrate the importance of TLR2 and TLR4 expression on radiosenstitive haematopoietic cells for an adequate antibacterial defence during *Klebsiella* pneumonia.

Consistent with earlier experiments (table 1), pulmonary levels of KC and IL-1β were reduced in H-/S- mice in comparison with H+/S+ mice, despite higher bacterial loads (table 2). Mice with TLR2- and TLR4-deficient stroma (S-) demonstrated reduced KC levels regardless of the origin of reconstituted haematopoietic cells (H+ or H-), implying KC is mainly produced by stromal cells, such as epithelia.

DISCUSSION

K. pneumoniae is a clinically important Gram-negative pathogen in hospital-acquired pneumonia. Previous research has demonstrated that TLR4 is important for an adequate host defence in *K. pneumoniae* pneumonia [4, 5, 7]. Given that *Klebsiella* expresses several TLR2 ligands [3, 11], we sought here 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 and TLR4 on haematopoietic and stromal cells. 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 and TLR4 expressed by radiosensitive haematopoietic cells and not by radioresistant stromal cells are important for an adequate host response.

Klebsiella pneumonia and pneumosepsis are common in and out of the hospital environment [1, 2]. Nosocomial pneumonia mainly affects patients with pre-existing diseases that may impact on host defence in the lung. Our model uses previously healthy mice and, as such, more closely 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

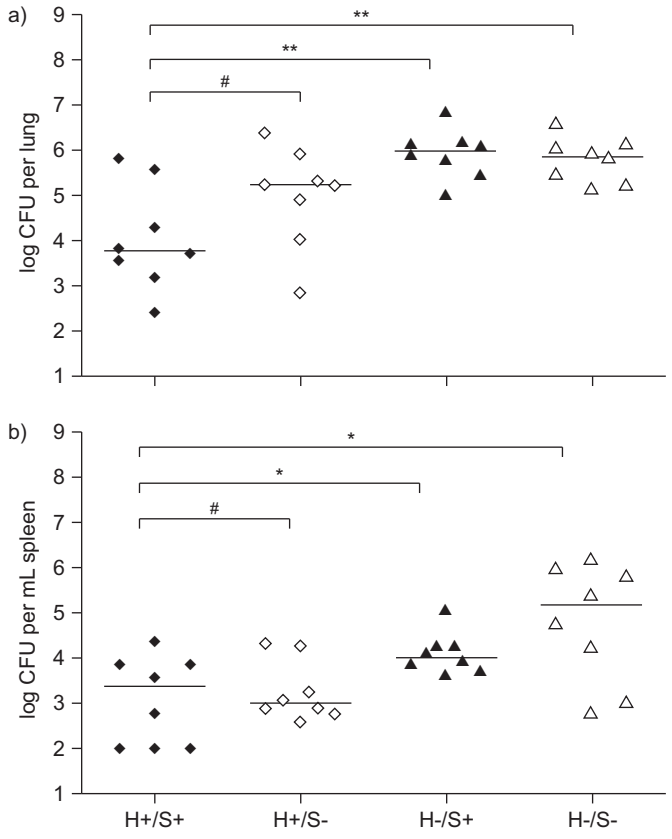

FIGURE 4. Toll-like receptor (TLR)2 and TLR4 expressed by haematopoietic cells are important for antibacterial defence during *Klebsiella pneumoniae* pneumonia. Wild-type (structural (S)+) and TLR2×4 knockout (KO; S-) mice were irradiated and injected with WT (haematopoietic (H)+) or TLR2×4 KO (H-) bone marrow cells. 6 weeks after transplantation, mice were infected with 10^4 colony-forming units of *K. pneumoniae* and sacrificed 24 h later. a) Pulmonary and b) splenic outgrowth of *K. pneumoniae* was determined in organ homogenates. Each symbol represents an individual mouse. —: median. ◆: H+/S+; ◇: H+/S-; ▲: H-/S+; △: H-/S- *: $p<0.05$ *versus* H+/S+; **: $p<0.01$ *versus* H+/S+; #: not significant.

in this study is a common laboratory strain that was used in many previous studies investigating host response against *K. pneumoniae*. 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 signalling was associated with upregulation of other receptors with mainly proinflammatory 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 defence. Indeed, relative to WT mice, TLR2 KO mice had higher bacterial counts in lungs 48 h (but not 24 h) after infection with $3x10^3$ CFU *Klebsiella*, whereas TLR2×4 double KO mice displayed higher bacterial burdens than TLR4 KO mice in lungs and distant organs 24 h after infection with 10^4 CFU *Klebsiella*, which was associated with an accelerated lethality beyond this time-point. TLR4 clearly played a more prominent role in antibacterial defence than TLR2, especially in the initial phase of the infection, as TLR4 KO (but not TLR2 KO) mice demonstrated enhanced bacterial growth in lungs and spleen 24 h after infection with 3×10^3 CFU *Klebsiella* when compared with WT mice, and TLR4 KO mice and TLR2×4 double KO mice harboured 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 realise 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

TABLE 2 Lung cytokine and chemokine levels in chimeric mice 24 h after infection

	KC pg·mL^{-1}	MIP-2 pg·mL^{-1}	IL-1β pg·mL^{-1}	IL-6 pg·mL^{-1}	TNF pg·mL^{-1}
H+/S+	11224±778	22822±11200	1949±472	859±156	1368±302
H+/S-	6115±1605*	32113±12403	3411±1122	1271±236	1944±648
H-/S+	11198±682	34712±11590	2674±490	1328 ± 176	1755±395
H-/S-	6954±1321*	4245±1035	855±154*	1106±129	1978±425

Data are presented as mean±SEM. Chimeric mice were infected with 4×10^4 colony-forming units of *Klebsiella pneumoniae* 6 weeks after bone marrow transplantation. 24 h later, mice were sacrificed, lungs were removed and keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF) were determined using ELISA. n=7–8 mice per group. H: haematopoietic; S: structural. *: $p<0.05$ *versus* wild-type levels (H+/S+).

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 defence against bacterial infections [21]. However, in addition to neutrophils and lung macrophages, nonhaematopoietic cells, such as lung epithelium and endothelium, contribute to the initial recognition of bacteria, production of inflammatory mediators and, thus, host defence. To dissect the role of haematopoietic and nonhaematopoietic cells, we generated bone marrow chimeric mice using WT and TLR2 × 4 double KO mice (the most susceptible mouse strain). We found that injecting WT bone marrow into irradiated TLR2 × 4 KO animals resulted in similar bacterial growth, as in syngeneic transplanted WT mice, and reduced outgrowth compared with syngeneic TLR2 × 4 KO animals, meaning that haematopoietic cells are of utmost importance in host defence. Consistent with this, irradiated WT mice that received TLR2 × 4 KO bone marrow did worse than syngeneic transplanted WT animals. As differences in antibacterial host defence between syngeneic transplanted (H+/S+ and H-/S-) mice on the one hand, and nontransplanted WT and TLR2 × 4 KO animals on the other hand were similar, we believe that our results are not an artefact introduced by the bone marrow transplantation procedure.

Several earlier studies investigated the role of TLRs in host defence against *Klebsiella* pneumonia. In accordance with the current data, C3H/HeJ mice (which harbour 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 interferon-β) and MyD88 [3], and both TRIF KO and MyD88 KO mice displayed an impaired host defence during *Klebsiella* pneumonia [9]. Notably, MyD88 mediates signalling of all TLRs excluding TLR3, and of the signalling 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 signalling 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 haematopietic cells interact in mediating an effective antibacterial defence 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 signalling 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.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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