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TLR4 is involved in mediating fatal murine pneumonia due to *Burkholderia cenocepacia*

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ABSTRACT

Background: We previously showed that MyD88 knocked out mice were protected from death due to *B. cenocepacia* pneumonia implying that a toll-like receptor(s) (TLR) was involved in mediating death. The aim of the present study was to determine which TLR(s) was involved in triggering the inflammatory response responsible for the pathogenesis. We specifically focus on the TLRs 4 and 5, as these two receptors are the main ones involved in the recognition of *P. aeruginosa*, a flagellated Gram-bacterium similar to *B. cenocepacia*. Methods: Mice were infected intratracheally with a suspension of *B. cenocepacia*. Animals were then observed daily for signs of morbidity. Alternatively, bronchoalveolar lavages (BAL) were collected at different time points to further determine cytokine concentrations and the number of CFU of *B. cenocepacia*. Results: The data clearly indicate that the innate immune response of the host to *B. cenocepacia* lung infection was due to TLR4 that senses the pathogen while TLR5 does not do so *in vivo*. As with the MyD88-/- strain, TLR4-/- mice were protected from death and cytokine and chemokine synthesis to infection were reduced. The only paradoxical observation was the reduced pathogen burden in the case of TLR4-/- mice compared to the enhanced (but transient) pathogen burden observed with MyD88-/- mice, suggesting that another TLR was involved in bacterial clearance. Conclusion: The data clearly demonstrate a deleterious implication of TLR4 in the host to *B. cenocepacia* lung infection.

Keywords: Rodent; Bacterial; Inflammation;

[#] These authors contributed equally to the work.

Innate Immunity; Lung

1. INTRODUCTION

Burkholderia cepacia complex (Bcc) strains have emerged as problematic opportunistic pathogens causing severe infections in patients with immune suppression, chronic granulomatous disease and cystic fibrosis [1-3]. Infections due to Bcc are specially a serious concern to CF patients due to their inherent antibiotic resistance and high potential for patient-to-patient transmission [4]. At least 17 Bcc species have been recovered from respiratory secretions of CF patients in many countries [5-6], but *B. cenocepacia* is the most common species isolated [5,7-8]. The clinical outcomes of Bcc infections range from asymptomatic carriage to a fulminant and fatal pneumonia, the so-called "cepacia" syndrome [9]. There is increasing evidence that the acute pulmonary deterioration associated with *B. cenocepacia* infection is the consesquence of a marked inflammatory host response [10]. This could be due to lipopolysaccharide (LPS) as it has been shown that LPS purified from Bcc species exhibits potent proinflammatory activity mediated through Toll-like receptor (TLR) 4 [11], and may account, at least in part, for the sepsis-like cepacia syndrome [10-12].

TLR recognize conserved pathogen-associated molecular patterns and trigger the activation of genes involved in innate defense. Four main TLRs are involved in sensing bacteria, TLR2 in tandem with TLR1 or 6 detects the presence of lipopeptides and peptidoglycan, TLR4 in association with CD14 and MD2 recognizes LPS, TLR5 detects the presence of flagellin, and TLR9 recognizes hypomethylated DNA [13-15].

Numerous groups have established the importance of TLRs in pulmonary host defense and this laboratory has demonstrated their involvement during influenza A [16], *Aspergillus fumigatus* [17,18], and *Pseudomonas aeruginosa* [19-21] infections. Recently, we showed that

MyD88, a key downstream adapter for most of the TLRs, was involved the death due to *B. cenocepacia* pneumonia suggesting a role of TLRs in the pathogenesis [22]. Thus, MyD88 knocked out mice (MyD88-/-) were protected from death following *B. cenocepacia* lung infection. We demonstrated that the pathogenesis was due to a hyperinflammatory host response mediated by TNF-α. The aim of the present study was to determine which TLR(s) is playing a role in the recognition of *B. cenocepacia* and triggering of hyperinflammation. We specifically focus on the TLR4 and 5, knowing that these two receptors are the main actors in the recognition of *P. aeruginosa*, a flagellated Gram- bacterium which like *B. cenocepacia* is a highly problematic pathogen for individual with CF [19,20,23-25].

2. METHODS

2.1. Bacterial Strain and Growth Conditions

B. cenocepacia of the epidemic ET12 lineage (strain J2315) was provided by the Pasteur Institute microorganisms depository. Bacteria were grown on tryptic soy agar (TSA) at 33°C for 48 h. Single colonies removed from the plate were grown in 5 mL of tryptic soy broth at 33°C with shaking for 16 h - 18 h, corresponding to midlog phase. Bacteria were harvested by centrifugation (3,000 ×*g* for 15 min), resuspended in saline and optical densities of the suspensions were adjusted to give the desired bacterial concentration which was verified by serial dilutions and plating on TSA.

2.2. Mouse Strains

TLR4-/- and TLR5-/- mice were obtained from S. Akira (Osaka University, Osaka, Japan). All mice were backcrossed at least eight times with C57BL/6 to ensure similar genetic backgrounds. Double knocked-out TLR4,5-/- mice were generated by breeding TLR4-/- mice and TLR5-/- mice. C57/BL6 mice from which these mice were derived were used as the control mice. These latter mice were supplied by the Centre d'Elevage R. Janvier, Le Genest Saint-Isle, France and used at about 8 weeks of age. Upon arrival for experimentation mice were fed normal mouse chow and water *ad libitum* and were housed under standard conditions with air filtration. Mice were cared for in accordance with Pasteur Institute guidelines in compliance with the European animal welfare regulation.

2.3. Immunosuppressive Treatment and Infection

As mice are relatively resistant to *B. cenocepacia* and generally do not die from pulmonary infection unless encased in agar beads, we developed a lethal model in the neutropenic mouse [22], where wild type mice die after an intratracheal challenge with about $4 - 5 \times 10^7$ cfu. Chemotherapy-induced neutropenia was achieved by the intravenous administration of 5 mg/kg of the antineoplastic drug vinblastine (Cell Pharm GmbH, Germany) 66 h before infection. Under these conditions, the haematological profile of the mice after vinblastine and during the infection displayed total polymorphonuclear cell depletion from day 0 (the day of infection) until day 2 for both wild-type and TLR-/- mice. Of note, in separate investigations, we previously demonstrated that with this vinblastine regimen, mouse survival and cytokine production are similar to those obtained when neutropenia is induced with the antigranulocyte monoclonal antibody RB6-8C5, [26]. Animals were infected intratracheally under general anesthesia achieved with a mixture of ketamine (40 mg/kg) and xylazine (8 mg/kg) administered *via* the intramuscular route and infected as previously described [19] with a 50-µl suspension of *B. cenocepacia* suspension (4×10^7 cfu/mouse). Mice were then observed daily for signs of morbidity. Alternatively, mice were killed at different time points (6 h or 24 h) by intraperitoneal injection of 300 mg/kg sodium pentobarbital. Airways were washed twice with 1 ml saline, and the bronchoalveolar lavage (BAL) was collected [27] to further determine cytokine concentrations using DuoSet ELISA kits (R&D Systems). One hundred microliters of the BAL were diluted and plated on TSA plates to determine the number of CFU of *B. cenocepacia*.

2.4. Statistics

Survival of wild-type and TLR-/- animals was compared using Kaplan-Meier analysis log-rank test. Inflammatory mediators levels and bacterial counts were expressed as the mean ± SEM. Differences between groups were assessed for statistical significance using the Kruskal-Wallis ANOVA test, followed by the Mann-Whitney *U* test. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Effect of TLR4 and TLR5 Deletion on Mouse Survival

The survival of WT, TLR4-/-, TLR5-/- and TLR4,5 double knockout neutropenic mice was followed for 10 days following intratracheal challenge with *B. cenocepacia*. The survival curves are shown in **Figure 1**. It was noted that only 22% of WT mice survived during the 10 day observation whereas 84% of the mice that had TLR4 deleted ($p < 0.0001$ compared to WT), and 75% of the
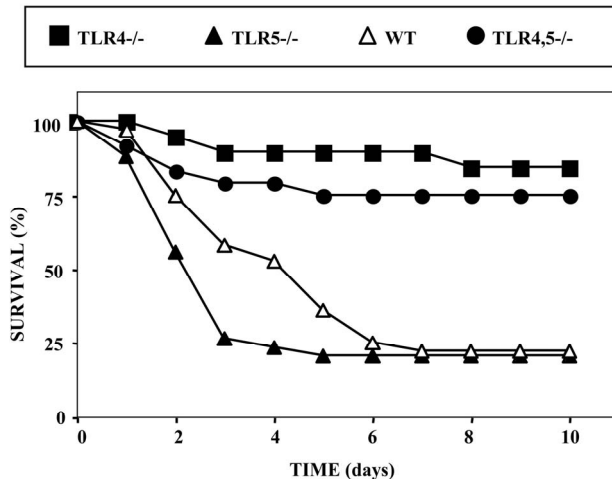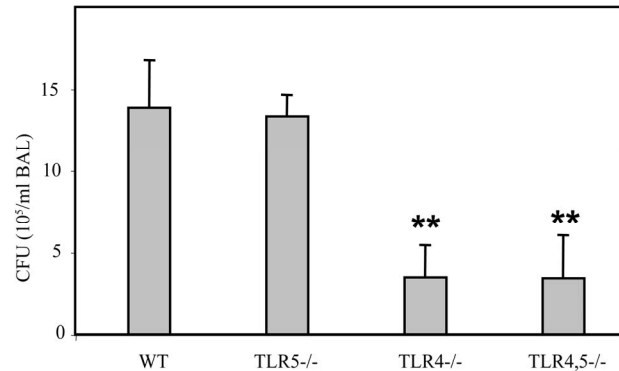
Wild-type, TLR5-/-, TLR4-/- and TLR4,5-/- mice treated with vinblastine and infected by 4×10^7 cfu/mouse *B. cenocepacia*. Survivals were checked every 24 h. Logrank (Mantel-Cox) test for comparisons of Kaplan-Meier survival curves indicated a significant difference in the survival of TLR4-deficiente mice (n = 19 for TLR4-/-; $p < 0.0001$; and n = 24 for TLR 4,5-/-; $p < 0.0005$) and no significant difference in the survival of TLR5-/- mice (n = 34 for TLR5-/-; $p > 0.05$) compared to that of wild-type animals (n = 36).

Figure 1. Mice survival upon infection by *B. cenocepacia* as a function of TLR4 or 5 expression.

mice that had both TLR4 and TLR5 deleted ($p < 0.0005$ compared to WT) survived. Mortality among TLR5-/- mice was non significantly different from that of WT mice, both in terms of percentage mortality and time to death. Collectively, these data suggested that the TLR4 response to *B. cenocepacia* was indeed deleterious as has been noted for the response to LPS challenge [10,12] and that the TLR5 response did not play a significant role either in protecting the mice or inducing a deleterious inflammatory response.

3.2. Role of TLR in the Clearance of *B. Cenocepacia* from the Airways

In order to examine whether there was a correlation between bacterial clearance, survival and TLR function, we examined bacterial clearance from the airways of WT and mutant mice by culturing of BAL fluid (**Figure 2**). At 24 h hours post-infection, both WT and TLR5-/- mice had retained similar amounts of the challenge dose. Thus, clearance of this bacterium did not appear to be affected by the absence of a TLR5 response, which was not surprising as the TLR4 arm of the innate immune response was intact. However, the number of bacteria remaining in the airways of TLR4-/- ($p < 0.01$) and TLR4,5-/- ($p < 0.01$) was significantly lower than that seen in WT mice despite the latter mice not having the two major TLRs that are involved in defense against flagellated gram negative bacteria. Thus the response mediated through TLR4 appears to both lead to death

Wild-type, TLR5-/-, TLR4-/- and TLR4,5-/- mice were treated with vinblastine and infected by 4×10^7 cfu/mouse *B. cenocepacia*. BAL were performed at 24 h p.i. for the measurements of bacterial loads determined by serial dilutions and plating on TSA. Data are the mean ± SEM values obtained from four animals. **, $p < 0.01$ when compared with the corresponding WT values. ns = non significant.

Figure 2. Pathogen burden of the lung upon infection by *B. cenocepacia* as a function of TLR4 and 5 expression.

and to inhibit bacterial clearance, since in its absence there is significantly better survival and bacterial clearance from the airways compared to WT mice. This finding is consistent with enhanced survival seen with MyD88-/- mice [22] where multiple MyD88-/- dependent TLRs are not functional. One salient difference however is that the bacterial burden in MyD88-/- mice at 24 h was much greater than that seen in WT mice [22], suggesting that another MyD88 dependent factor, active in TLR4, 5-/- mice, functions to assist in the early clearance of *B. cenocepacia*.

3.3. Role of TLR in the Induction of Cytokine/ Chemokine Secretion during *B. Cenocepacia* Lung Infection

Using the BAL fluids collected to examine bacterial clearance from the airways, we measured several cytokines/chemokines responses of the mice to explain the sometimes paradoxical data obtained for survival and bacterial clearance. TNFα, which we had previously shown to be involved in the death of mice infected with this bacterium [22], was significantly lower in TLR4-/- mice than in WT mice but just as high in TLR5-/- mice as in WT mice (**Figure 3**), confirmaing a significant role for this cytokine in LPS induced inflammation. Similarly all other cytokines/chemokines measured showed low levels in the absence of TLR4 and similar levels in WT and TLR5-/- mice (**Figure 4**).

4. DISCUSSION

The innate immune response of the host to *B. cenocepacia* lung infection clearly indicates that TLR4 senses the pathogen while TLR5 does not do so *in vivo*. In the absence of the former, the host response in terms
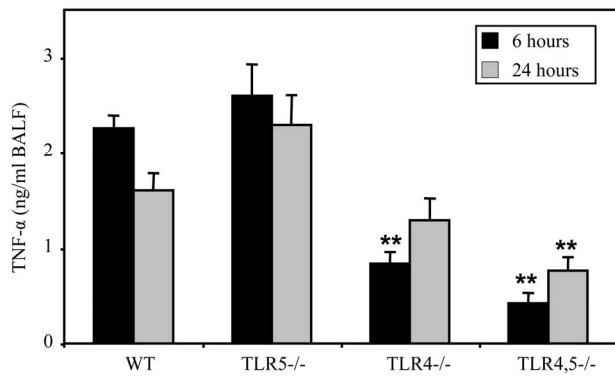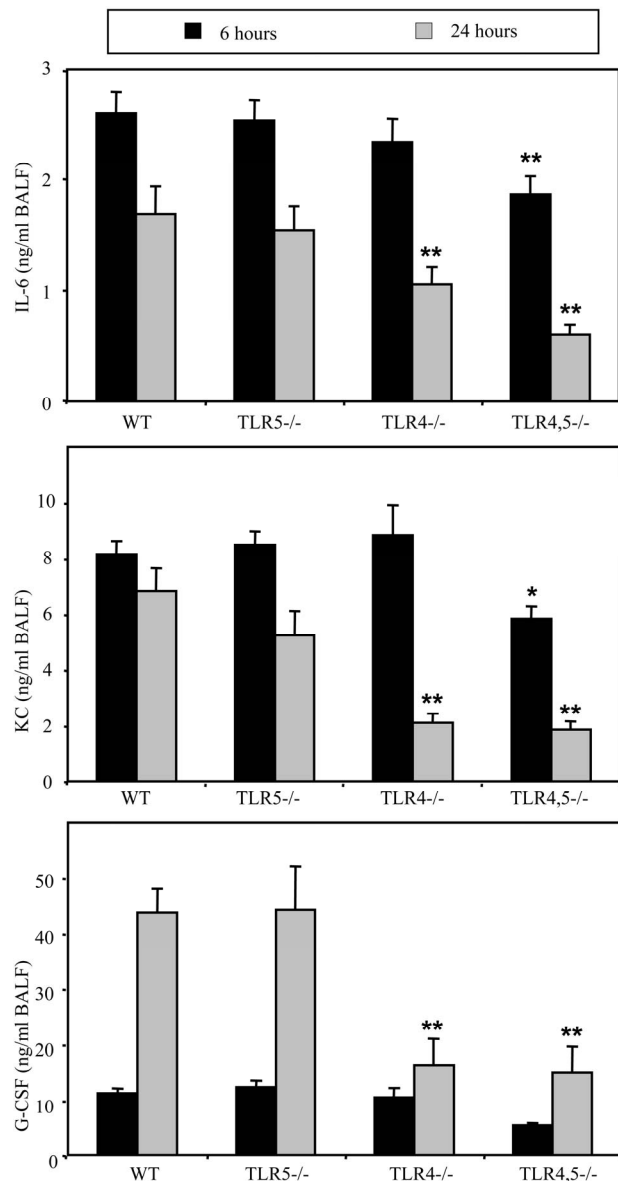
Wild-type, TLR5-/-, TLR4-/- and TLR4,5-/- mice were treated with vinblastine and infected by 4×10^7 cfu/mouse *B. cenocepacia*. BAL were performed at 6 and 24 h p.i. for the measurements of TNF-α concentrations. Data are the mean ± SEM values obtained from eight animals. **, $p < 0.01$ when compared with the corresponding WT values. ns = non significant.

Figure 3. Induction of TNF-α synthesis upon infection by *B. cenocepacia* as a function of TLR4 and 5 expressions.

of cytokine and chemokine synthesis to infection is reduced, although not totally ablated, indicating the participation of other signaling pathway(s). Moreover, as with the MyD88-/- strain, TLR4-/- mice were protected from death. We thus deduce that activation of TLR4 by *B. cenocepacia* triggers a hyperinflammatory state that is responsible for the observed pathogenesis [22]. The only paradoxical observation is the reduced pathogen burden in the case of TLR4-/- mice compared to the enhanced (but transient) pathogen burden observed with MyD88-/- mice. How TLR4 inhibits bacterial clearance was not ascertainable, but the observation appears to be consistent for the two different groups of mice with the TLR4-/- deletion. It is also of note that the *in vivo* data do not fit with our previous *in vitro* data [28]. Using respiratory epithelial cells isolated from TLR4-/- mice or cells over-expressing a functional form of TLR5, we established that TLR5, but not TLR4, mediated *B. cenocepacia*-induced lung epithelial inflammatory response. Such a discrepancy between *in vivo* and *in vitro* results suggests that the role of respiratory epithelial cells in lethal *B. cenocepacia* pneumonia is limited. We have however previously noted differences between *in vivo* and *in vitro* data. Thus, with *P. aeruginosa*, we observed the lack of involvement of TLR2 and 4 in the induction of IL-6 synthesis *in vivo* [19] while the absence of expression of the very same TLRs by respiratory epithelial cells infected *in vitro* reduced the synthesis of IL-6 considerably [21]. Altogether, it is clear that examination of epithelial cells in tissue culture is prone to variations and may not always be reflective of infection.

The observations reported here also do not concur with those of Urban *et al.* [29] who used an agar bead model of infection to examine the role of flagella in *B. cenocepacia* chronic lung infection. They observed reduced mortality

Wild-type, TLR5-/-, TLR4-/- and TLR4,5-/- mice were treated with vinblastine and infected by 4×10^7 cfu/mouse *B. cenocepacia*. BAL were performed at 6 and 24 h p.i. for the measurements of IL-6, KC and G-CSF concentrations. Data are the mean ± SEM values obtained from at least eight animals. *, $p < 0.05$ and **, $p < 0.01$ when compared with the corresponding WT values. ns = non significant.

Figure 4. Induction of IL-6, KC and G-CSF synthesis upon infection by *B. cenocepacia* as a function of TLR4 and 5 expressions.

and noted significant reductions of KC, a murine homolog of IL-8 both in BAL fluid and serum, when wild type mice were challenged with a flagellin mutant, indicating that there is normally a TLR5 mediated response in that model of infection. However, their data *in vitro* showing that flagellin does stimulate a response in cells through TLR5 is similar to ours [28]. Rather than dismiss a role for TLR5, this discordance raises the ques-

tion whether *B. cenocepacia* expresses flagellin *in vivo* during an acute infection versus the agar bead model of infection. This possibility is not with precedent, as members of the genus *Bordetella*, which are responsible for a variety of acute bronchial infections repress flagellum production upon entry into the airways [30]. Lastly, while we have chosen to examine the role of LPS mediated inflammation in death, it is likely that other virulence factors may also be involved, as some TLR4-/- mice do still die. This organism is known to have at least four protein secretion systems, any or all of which may also be involved in death. Analysis of these factors is however beyond the scope of this study.

It is concluded that *B. cenocepacia* induces a hyperinflammatory state mediated through its very potent LPS [10,12]. However, suppression of the LPS-TLR4 interaction and the consequent down-regulation of lung inflammation still leaves an effective innate immune response that is capable of controlling bacterial growth.

5. ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS USED

BAL, bronchoalveolar lavage;
CF, cystic fibrosis;
MyD, myeloid-differentiation;
p.i., post-infection;
TLR, Toll-like receptor.