Author's response to reviews

Title: The role of TLR2 in the host response to pneumococcal pneumonia in absence of the spleen

Authors:

Adriana J.J. Lammers (a.j.lammers@amc.uva.nl)
Alexander de Porto (alexanderdeporto@gmail.com)
Onno de Boer (o.j.deboer@amc.uva.nl)
Sandrine Florquin (s.florquin@amc.uva.nl)
Tom van der Poll (t.vanderpoll@amc.uva.nl)

Version: 2 Date: 18 December 2011

Author's response to reviews: see over
Dear Editor,

Thank you for considering our manuscript “The role of TLR2 in the host response to pneumococcal pneumonia in absence of the spleen” (Manuscript ID 1981109480561913) for publication in BMC Infectious Diseases.

We hereby send you our responses to the reviewers comments.

Best regards,

Jolanda Lammers
Reviewer 1: Xavier Wittebole

GENERAL COMMENT:
The question addressed in the manuscript is: ?Does an intact spleen compensate for TLR2 deficiency during pneumococcal pneumonia?? To answer this question, the authors report on the effect of TLR2 and, or, TLR4 deficiency in splenectomized mice intranasally infected with 2 different strains of streptococcus pneumoniae. They demonstrate in splenectomized mice an absence of significant role for TLR2 and TLR4 in host defense against S. pneumoniae. Indeed, mortality, bacterial loads in lung and blood, cytokine lung levels and lung histological alterations, were equivalent in splenectomized wild type animals and in TLR2 KO mice, whatever the dose of pneumococcus used. The double KO (TLR2 and 4) mice display the same kind of result. Together those results suggest a non-significant role for TLR2 and TLR4 in splenectomized animals infected with S. pneumoniae.

MAJOR COMMENTS:

1. The hypothesis of work report on an intact spleen (abstract). However, all groups studied in this manuscript report on the effect on TLR2 and TLR4 deficiency in the absence of spleen. This is somewhat contradictory with the hypothesis. To study the hypothesis, 4 groups of animal should be evaluated (spleen+/TLR2 +, spleen+/TLR2 -, spleen -/TLR2 +, spleen -/TLR2 -). Do the authors have those data? Otherwise, they should rephrase their hypothesis.

Our laboratory previously reported on the course of pneumococcal pneumonia in TLR2 KO mice with an intact spleen (please see: Knapp S et al. J Immunol 2004, 172:3132-3138 and Dessing MC et al. Cell Microbiol 2008, 10:237-246; references 8 and 12 of the current manuscript), showing that TLR2 does not contribute to host defense in these animals. Therefore, we did not repeat these experiments in the present investigation. This is clarified in the manuscript as follows:

Abstract (page 2): “Although Toll-like receptor (TLR)-2 is considered the major receptor for Gram-positive bacteria in innate immunity, it does not play a major role in host defence against pneumococcal pneumonia. We wanted to investigate if in absence of an intact spleen as a first line of defence, the role of TLR2 during pneumococcal pneumonia becomes more significant, thereby explaining its insignificant role during infections in immune competent hosts”.

Introduction (page 3): Please see second paragraph, which is entirely devoted to the role of TLR2 in host defence during pneumococcal pneumonia in mice with an intact spleen.

2. As reported in the manuscript (page 3 line 19 and further) TLR2 does not seem to play a major role in host defence against pneumococcal pneumonia (reference 8,11,12). Therefore, the authors should explain why they thought TLR2 could have any effect on the various outcome studied after pneumococcal pneumonia in the absence of spleen, an explanation can be found in the latter part of the discussion but this should be described in the introduction, since it is related to the hypothesis.

We have rewritten the introduction as suggested by the reviewer, see high-lighted paragraph in the Introduction section, page 4-5.
3. The authors specifically address the problem of TLR2 and TLR4 in this manuscript. We now know that TLR 9 is of particular importance (Albiger B et al. Cell Microbiol. 2007; reference 11) as is MyD88 (Albiger B et al. Cell Microbiol. 2005). Why didn’t the author study TLR9KO mice or MyD88 KO mice?

We did not perform studies in MyD88 or TLR9 KO mice after splenectomy. We here focused on TLR KO mice without a clear phenotype in this model of pneumococcal pneumonia in the presence of an intact spleen (as the referee correctly notes MyD88 and TLR9 KO mice have been described to be more susceptible to pneumococcal pneumonia). We agree that studies using MyD88 and TLR9 KO mice are of interest for future studies. To clarify this in the revised manuscript we added the following paragraph to the Discussion (page 15, see high-light):

“Previous studies have implicated TLR9 and MyD88 as important players in protective immunity in pneumococcal pneumonia (Albiger et al. Cell Microbiol 2005 and 2007). We here focused on the role of TLR2 in defence during S. pneumoniae pneumonia in the asplenic host, considering that this TLR does not play a significant part in limiting bacterial growth in animals with an intact spleen (references 8,11,12). Future studies are warranted to investigate the role of MyD88 and TLR9 in asplenic animals during respiratory tract infection caused by the pneumococcus”.

4. Pneumolysin was demonstrated to act through TLR4. Therefore the authors studied the effect of TLR4 in splenectomized animals. However, pneumolysin also acts through the nlrp3 inflammasome, in a TLR4 independent manner (McNeela et al. PlOx Pathog. 2010; Witzenrath M et al. J Immunol. 2011). A comment should be added to the manuscript.

In accordance with the suggestion of the reviewer we added the following section to the Discussion (page 14, see highlights):

“We did not investigate non-TLR signaling in this model. Recently, it was shown that human and murine mononuclear cells respond to S. pneumoniae expressing pneumolysin by producing IL-1β via a mechanism that depended on the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome. Specifically, release of IL-1β was induced by wild-type D39 S. pneumoniae but not by pneumolysin-deficient pneumococci [36], showing a TLR-4 independent route of pneumolysin signaling”.

5. The authors conclude their discussion with a suggestion (?TLR2 mediated immune response might be dependent on the spleen as an effective organ:?). As described in comment #1, this could be answered by studying the above-mentioned groups of animals. Please see response comment 1.
Reviewer 2: Frederic Pene

The authors assessed the role of TLR2 in splenectomized hosts subjected to a pneumococcal pneumonia. The question is of interest to understand the physiological role of TLR2 in pneumococcal infections. Indeed, though TLR2 is clearly involved in sensing S. pneumoniae, it appears quite dispensable in non-immunocompromised hosts subjected to pneumococcal infections. In this straightforward paper, the authors concluded that TLR2 is also dispensable in the defence of splenectomized animals. Thus, the own role of TLR2 in pneumococcal infections remains questionable. I have several comments:

**Major comments**

1. The main goal of the study was to investigate if an intact spleen can compensate for a TLR2 deficiency, or, in other terms, to evaluate the protective role of TLR2 in the setting of splenectomy. The authors used a bacterial load known to be innocuous for non-splenectomized animals, and it appears clearly that this dose is highly lethal for both WT and TLR2 KO splenectomized animals. Although this model clearly shows that splenectomized animals are highly susceptible to pneumococcal pneumonia, it is not appropriate to reveal a higher susceptibility of TLR2-deficient mice. The authors should rather use a non lethal or sublethal model of pneumococcal pneumonia in TLR2-sufficient mice without spleen, and then check whether TLR2-deficient mice are more susceptible or not to the challenge.

   For the current experiments we specifically chose an infectious dose that results in a survival of approximately 50% at 48 hours. Since overwhelming pneumococcal infection after splenectomy in humans causes irreversible infection leading to mortality within the first 48 hours, we chose to study the role of TLR2 within this relevant period using bacterial loads to cause severe disease without killing the mice before predefined time-points of euthanasia. In the current model, all mice survived the first 24 hours after inoculation. We therefore think that our data provide information that is relevant for the clinical syndrome of severe pneumococcal infection after splenectomy. Nonetheless, we agree with the reviewer that our data do not exclude a role for TLR2 in asplenic animals after infection with a very low nonlethal dose of *S. pneumoniae*.

   To discuss the issue raised by the referee, we added the following to the Discussion (page 15, see highlighted paragraph):

   “We used an infectious dose that caused lethality in virtually all mice beyond the 48 hour time point. We specifically chose this dose considering that overwhelming pneumococcal infection after splenectomy in humans causes irreversible infection leading to mortality within the first 48 hours (references 17,18,37). As a consequence, our data do not exclude a protective role for TLR2 in asplenic animals after infection with a low nonlethal dose of *S. pneumoniae*”.

2. Studies that assessed the role of the spleen in pneumococcal infections were performed using bacteremia models which are not the physiological route of infection in most *S. pneumoniae* infections. So it is likely that the spleen might rather prevent systemic spread of the disease, and blood bacterial loads should also be displayed as scatterplots.

   In accordance with the suggestion of the reviewer we added blood bacterial loads to the figures of the manuscript (please see revised figures, 3 and 5).
3. I would be interested in having the survival curve of WT and TLR2KO mice subjected to serotype 3 S. pneumoniae pneumonia, and of splenectomized TLR2/4 -/- mice subjected to serotype 2 S. pneumoniae pneumonia.

We agree that these survival curves would be of interest. However, the institutional Animal Care and Welfare Committee does not allow large scale survival studies, especially in experiments wherein bacterial loads do not differ between groups. We specifically asked for approval for the survival study shown in figure 1a. In light of similar outcome data with regard to bacterial loads in the other experiments, approval was not granted for additional survival studies.

Additional comments
1. There is a trend towards decreased histological inflammation in TLR2-/- mice. This mentioned in the discussion section but not reported in the results section. Text in Results section has been adjusted (see high-lighted sections, page 10 and 11).

2. Since the number of animals per point is low, the boxplot distribution of variables is not relevant. Data should be displayed as scatterplots. We have adjusted the figures to the reviewers suggestion.