Author's response to reviews

Title: Adjuvant TACE inhibitor treatment improves the outcome of TLR2/- mice with experimental pneumococcal meningitis

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Author's response to reviews: see over
Comment Nr.1:
In their discussion the authors state that bacterial load and TNF levels in CSF are major determinants for outcome. A problem with these studies in my opinion is that the authors didn't mention anything about the effect of the treatment with TACE inhibitor on the number of leukocytes in the CSF, a major hallmark of bacterial meningitis.

Answer:
We have inserted the development of leukocyte numbers in CSF in the four mouse strains and shown the data in Fig. 2C with legends on p.18 and text on p. 9. We have described the effect of TACE inhibition upon leukocytes on p. 10 line 9.

Comment Nr.2:
A problem with these studies in my opinion is that the authors didn't mention anything about the effect of the treatment with TACE inhibitor......on the production of other cytokines and chemokines (in brain homogenates) which also are very important determinants for outcome.

Answer:
We have performed new measurements of MMP-9 in brain homogenates from mice with and without TACE inhibitor treatment and we have described the effect of TACE inhibition upon MMP-9 on p. 10 line 9. The method of MMP-9 determination is described on p. 7.

Comment Nr.3
Also histopathology of the brain of the various mice before and after treatment will give important information about the effect of TACE inhibitor on meningeal inflammation.

The anti-inflammatory effects of TACE inhibitor have been described on p. 10; the measurement of leukocyte numbers and TNF in CSF, and of MMP-9 in brain homogenates respectively have been included. The histopathological scoring of meningitis in HE-stained sections does not give a more precise quantitative information on the effect of the adjuvant anti-inflammatory treatment than the enumeration of leukocytes in CSF, as we have performed and described (see comment Nr. 2). Histopathological analysis has been performed in CD14⁺ mice and published to illustrate the extent of leukocyte infiltration (Echchannaoui et al. J Leukocyte Biol. 78, 705, 2005).

Minor revisions:
-page 14, s. 9: related, the text has been corrected.
Comment Nr.1
Efficacy of antibiotic or TACE inhibitor treatment on S.P.-induced experimental meningitis in otherwise normal mice. These data are presented in the figures of this manuscript as a comparison with novel data related to the use of double KO mice. However, the double KO mice have not been used for the pharmacological experiments where only TLR2 and CD14 KO were studied.

Answer:
Double TLR2/CD14 KO mice WERE treated with antibiotics (Fig. 1B), but since they were cured with antibiotics alone, they were not treated with TACE inhibitor.

Comment Nr.2:
Therefore, I suggest that the data shown in figure 1 and 2 should be deleted and simply mentioned in the text.

Answer:
The survival curves as shown here in Fig. 1 from 4 mouse strains (C57BL/6, TLR2^{-/-}, CD14^{-/-} and TLR2^{-/-}/CD14^{-/-} mice) in one plot are important information for the reader. First, results from TLR2^{-/-}/CD14^{-/-} mice (Fig. 1A-C), are shown here for the first time and can only be interpreted, if shown in comparison with the other strains. Second, results of antibiotic treatment (Fig. 1C) have to be shown; they were not published for CD14^{-/-} and TLR2^{-/-}/CD14^{-/-} mice before and are valid in comparison with the TLR2^{-/-} mice. These observations form the argument to use TACE inhibitor in TLR2^{-/-} mice only.

Comment Nr.3
Figure 2 as it stands now shows bacterial load (A) and TNF measurements (B) in the total population of mice. However, one can desume from Fig 1 panel A that only part of the mice show the high severity score particularly after 24 h. To link bacterial load and high TNF in CSF to disease progression it would be important to divide the measurements of these 2 parameters in the 2 population of mice, namely the ones developing an accelerated disease progression vs the others.

Answer:
The relationship between the amount of inflammation and outcome in pneumococcal meningitis was published for the first time by Mc Allister in 1975 (JID, 132, 355). We published earlier in Journal of infectious Diseases (Echchannaoui et al. 186, 798, 2002) an association between disease severity and meningeal inflammation by showing a significant relationship between TNF in CSF and severity score 24h after infection (see fig. a below, R= 0.589, P<0.001).

We included a sentence on p. 4, line 7 to document that TNF was related to disease severity.
Comment Nr.4
It is not clear why the authors used TLR2 and CD14 KO mice:

Answer:
We and others previously showed that TLR2 and CD14 contribute to host defense in pneumococcal meningitis. For TLR2, results of our and other groups are described on p. 4, paragraph 2, lines 3-10. For CD14 the role in host defense - as observed and published by using CD14KO mice in pneumococcal meningitis -, is described on p. 5, paragraph 2.

Comment Nr.5
The differences between TLR2 and TLR2/CD14 KO mice should be explained more convincingly. I would expect to find the effects observed in TLR2 KO mice potentiated in double KO mice.

Answer:
As described in the introduction, as shown in figure 2 and discussed on p. 13, the contribution of TLR2 and CD14 singly to host defense in meningitis were found different, their roles were shown by using TLR2 and CD14KO mice respectively. TLR2 was found enhancing early bacterial clearance, accelerating leukocyte immigration and downregulating TNF; and CD14 was found downregulating leukocyte infiltration.

In addition, we have found as shown in the figure b below (unpublished results), that TLR2 and CD14 are not always coexpressed. TLR2 was expressed in blood PMN and remained unchanged in CSF PMN after infection. In contrast, CD14 was weak in resting blood PMN. After transmigration through the blood-brain barrier, PMN in the CSF acquired CD14 on their surface. Neither TLR2 nor CD14 were found in any other brain cells ((Cauwels et al., 1999) and unpublished observations). The question remains, whether for the modulation of inflammation in CSF, TLR2 and CD14 functioned together or independently. We found that they had no synergistic or additive effects; since TLR2\(^{-/-}\)/CD14\(^{-/-}\) mice had similar or lower TNF values and leukocyte numbers as wt mice; the effects of TLR2 and CD14...
were rather opposing. TLR2 was probably required for a normal transmigration, whereas CD14 might have downregulated infiltration. These conclusions are drawn from slowed leukocyte infiltration in TLR2\(^{-/-}\) mice, and strong early pleocytosis in CD14\(^{-/-}\) mice. Our conclusions on the role of TLR2 and CD14 combined is described on p.14, paragraph 2, last line.

**Fig.b revision**

**Minor revisions:**
1. Why the treatments were stopped after 4 or 5 days while mice were observed for 9 days? Why the antibiotic treatment was longer than TACE inhibitor treatment?
   **Answer:**
   The treatment schedule was followed as used in earlier studies for antibiotics (Echchannaoui et al. 2002) and for TACE inhibitors (Leib et al, 2001), as these regimens were sufficient to rescue mice.
2. How motor activity was assessed in mice?
   **Answer:**
   Exploration of the cage after setting the mice into a new cage was evaluated.
3. Were the mice killed at the various times indicated AFTER S.P. inoculation? Please clarify (p. 7, line 6).
   **Answer:**
   Text has been clarified.
4. Statement in Discussion at p. 12, line 10 are not supported by the data since double KO mice have the same rate of survival as CD14 KO mice but they do not show any increase in TNF alpha.
   **Answer:** arguments have been exchanged,
5. Fig. 1c: the application of antibiotics has been indicated in the legend, p.18.

### Expression of TLR2 (PE, FL2) /CD14 (FITC, FL1) or isotype staining with isotype control antibodies in blood PMN (left) and in CSF 24 h after infection (right) of wt, TLR2\(^{-/-}\), CD14\(^{-/-}\) and TLR2\(^{-/-}\)/CD14\(^{-/-}\) mice. One out of 3 similar experiments is shown.