25/04/2016

Dear Fang-Fang Ji, Scientific Editor,

Thank you for the review and your reply concerning our manuscript entitled:

"Anti-CD163-dexamethasone conjugate inhibits the acute phase response to lipopolysaccharide in rats"

Manuscript NO 25089

We have revised the manuscript according to the reviewer's helpful suggestions, and we feel that this has improved our paper.

Below follow point-to-point responses to the review comments:

Reviewer 02860895

Comment 1:
Why did free dexamethasone, irrespective of its dosage, induce an unfavorable effect?

In our study, only the high dose free dexamethasone induced an unfavourable effect. Glucocorticoids are well-known for their serious systemic metabolic side effects as they bind to the ubiquitous intracellular glucocorticoid steroid receptor present in most cell types. After administration of dexamethasone the spleen is known to undergo a corticosteroid-induced weight reduction due to lymphocyte loss and in our study we did indeed observe a reduced spleen weight in the group treated with high dose free dexamethasone as evidence of it's systemic effect. Spleen weight was unchanged in the low dose free dexamethasone (matching the conjugate dose) and the anti-CD163-dexa conjugate groups and so both regimens had no evident systemic side effects.

Comment 2:
A more critical problem is that an efficacy of dexamethasone-conjugated anti-CD163 was significant in comparison with LPS + low dose dexamethasone but not with controls. Does this result really indicate the efficacy of the antibody?

This is an important point. We believe it is appropriate to compare the effect of the anti-CD163-dexa conjugate with the low dose of dexamethasone as this dose is the same steroid dose. When comparing the anti-CD163-dexa conjugate treated group to the controls trends towards reductions in plasma TNF-a, IL-6 and serum levels of α-2-macroglobulin were observed although these changes didn't reach statistical significance most likely because of huge deviations in concentrations often observed when measuring cytokine levels etc.

One could speculate if the effect of the anti-CD163-dexa conjugate was solely due to the binding of antiCD163 to the CD163 receptor and not due to the effect of the dexamethasone conjugated to the antibody. However, we have previously tested a lower dose of dexamethasone conjugated to the

same antibody (0.004 mg/kg) and found that the anti-inflammatory effect was less potent and therefore unlikely due to the binding of the antibody itself.

Reviewer 01221925

Comment 1:
In this experimental model the Ab conjugate was administered 24 hrs prior to the LPS infusion. This creates certain difficulties in terms of a potential clinical scenario. The problem for most treatments having to do with inflammation and sepsis modulation is that by the time they are provided it is already too late, as the process has already been set in motion. Could the authors please comment on how they envision a clinical scenario.

We fully agree that our 'prevention' model is not comparable to the clinical scenario with an established inflammatory process. However we chose to start our investigations of the conjugate effects from this point because we aimed at establishing a proof-of-concept position to motivate further studies on interference with on-going inflammation. We do believe our findings to be in support of this approach and we are now moving on with treating other animal models with experimental inflammatory disease with the conjugate. We have now added this information to the discussion section.

Comment 2:
Why were the time points of 2 hrs and 24 hrs post-administration? Were there other time points considered?

We measured plasma TNF- and IL-6 levels two hours post-LPS as these cytokines have a short half-life in the circulation and therefore would not be detectable after 24 hours. Based on literature and our previous studies (Thomsen KL et al, Am J Physiol Gastroenterol Liver Physiol 2013; 304: G680-6), we examined the rats 24 hours after LPS injection, as this is the time point at which the systemic acute phase response is fully activated with markedly increased serum levels of the positive acute phase proteins. We have earlier described the time courses of these changes and therefore we did not include them in this experiment.

Comment 3:
Could the authors elaborate a bit more on why the spleen was used as a measure of steroid systemic effects?

After administration of dexamethasone the spleen is known to undergo a corticosteroid-induced weight reduction due to lymphocyte loss (Rungruang T et al). We have also demonstrated this in a pilot study of systemic effects of dexamethasone in which prolonged treatment with high dose dexamethasone induced pronounced weight reduction of the spleen (Graversen JH et al). We also refer to our answer to reviewer 02860895's comment 1.

Comment 4:
A potential benefit of using a more targeted approach may be that you could limit the more systemic side-effects? Have the authors encountered evidence of that?

This is an important issue for the validation of the conjugate advantages. In the present study, we found an unchanged spleen weight in the anti-CD163-dexa conjugate group whereas high dose dexamethasone induced spleen weight reduction. Also, in a previous study using the conjugate, we found that high dose dexamethasone induced both a reduction in spleen and thymus weight as well as a suppression of cortisol levels (another evidence of systemic effects) whereas such changes were not seen in the conjugate-treated group (Graversen JH et al). We therefore believe the presented data to corroborate the assumption that the conjugate has less marked systemic steroid effects.

Comment 5:
The activation of some of the markers of acute phase inflammation (such as TNF and IL-6, which served in liver regeneration) are not completely harmful, as (part of their multiple roles) have the effect of a warning system as well as setting in motion some of the defense mechanisms. Down regulating them could potentially affect this balance.

Your comment contains a very important point and we agree that this could be a potential risk when using the conjugate to effect. Therefore, it is essential that further studies are conducted in relevant animal models where long term effects e.g. on survival can be investigated and undesired effects revealed. This has now been mentioned in the discussion section.

Reviewer 02440441

The authors used anti-CD163 antibody-dexamethasone conjugate to inhibit the acute phase response to lipopolysaccharide in rats, and found that the conjugate reduced TNF-α and IL-6 levels. Although CD163 expressed high on monocytes and macrophages, in this study, no evidence indicated that TNF-a and IL-6 were released only from macrophages. Please change the conclusion 'This supports the role of macrophages activation and indicates an anti-inflammatory potential of the conjugate' to 'This further indicates an anti-inflammatory potential of the conjugate in vivo'

We apologize for the unclear phrasing, which has now been changed in the abstract conclusion.

Reviewer 00069297

The reviewer believes that the contents would give significant information to the readers.

Thank you.

Reviewer 00181532

Major comment 1:
Overall, the study is well designed. There are multiple bugs present in text probably due to the format issue. Please correct them.

Thank you for pointing out these bugs, they have now been taken care of.

Major comment 2:
The decreased hepatic mRNA levels correlate with the decreased serum protein levels. Is there a reason why hepatic protein levels not checked?

We intended to understand the effects of the interventions at the level of gene translocation, and on the functional level. That is why we focused on mRNAs and circulating acute phase proteins. We might have measured also the hepatic gene protein products but we expected there would be no further information in these values – and the parallel changes in mRNAs and circulation proteins seems to point the same way.

Minor comments:
Abstract- Results: change lipopolysaccharide to LPS

Results: The sentence 'while no free dexamethasone dose had any effect on liver mRNA or serum levels of a2M' is not clear. Please rephrase the sentence.

Results: recommend changing the words 'The high dose therapeutic dexamethasone dose...' to 'The high dose dexamehtasone ...'

Conclusion: The study was aimed to study the conjugate on the hepatic acute phase response to LPS. In the first sentence, please mention that again.

Introduction- Second paragraph Line 3: change 'release of activated' to 'release from activated' Line 5: change 'steroid-like' to 'steroid-induced' Line 6: change 'high concentration' to 'higher concentration' Fourth paragraph Line 3: change 'post-LPS' to 'post-LPS exposure' Line 5: same as the line 3 comment

Materials and Methods Animals Line 5: Food intake and body weight 'were' registered at the beginning and 'at the' end of the week. Design Please be consistent with the unit 'mg/kg' in text

Results Body and spleen weight Line 2: recommend changing to 'The high dose dexamethasone decreased ...' TNF-a and IL-6 Line 2: Please rephrase the sentence such as 'There was a trend for reduced TNF-a ...' instead of using the word 'tended'. Line 4: The last sentence is not clear. You meant comparing to vehicle only. Plasma-alanine transferase and bilirubin delete 'at measurement'

Discussion Be consistent with the abbreviation 'a2M' (not a2MG) in text. Third paragraph The first sentence has grammatical error.

*Figure legends Please correct the bugs in text. Table 1 Use different denotations. It is confusing to see # and ## or * and ** in table.*

Thank you for all these fruitful suggestions in order to improve our paper. Changes have now been made throughout the manuscript.

Yours sincerely,

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