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Human-specific staphylococcal virulence factors enhance pathogenicity in a humanised zebrafish C5a receptor model.

Kyle D. Buchan, Michiel van Gent, Tomasz K. Prajsnar, Nikolay V. Ogryzko, Nienke W. M. de Jong, Julia Kolata, Simon J. Foster, Jos A. G. Van Strijp and Stephen A. Renshaw
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MS TITLE: Human-specific staphylococcal virulence factors enhance pathogenicity in a humanised zebrafish C5a receptor model.

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Buchan et al. engineer a humanised zebrafish line, where neutrophils express the human c5a receptor, a target of an array of S. aureus host-specific virulence factors.
Accordingly, they demonstrate that hC5aR expression by zebrafish neutrophils leads to increased susceptibility of the larvae to staphylococcal infection in irf8-KD condition.

This proves the principle that humanised zebrafish larvae can represent a valuable model in research also to study aspects of infectious diseases that are generally regarded as highly host-specific.

The manuscript is well-written and structured and represents a valuable advance in the field. However, I have a few suggestions for improvement, as I outline below.

Comments for the author

- Figure 2: membrane-bound expression of hsac5aR-clover: the figure 2C is not very clear. Perhaps a higher resolution image could confirm this more robustly.
Also, in the bottom-right part of the image, it seems that the cytosolic mcherry is outside the cell, as it would be outlined by clover. Probably this is due to the live imaging. However, if the cell is moving, this might also compromise the conclusions made on mcherry/clover not overlapping across the yellow line above.

- Figure 3B: Why does expression of hsaC5aR lead to neutrophils that are less capable to respond to dreC5a (which they should still sense via the endogenous dreC5aR)? The authors should comment on this. Also, it would be useful to include statistical information for groups injected with the same chemoattractant but expressing/non-expressing hsac5aR. This would also justify why the authors use two-way ANOVA, rather than one-way ANOVA.

- Related to the above, could the authors show, or refer to studies, reporting that hsac5aR/dreC5aR is expressed by human/zebrafish neutrophils, and how for example this differs in levels from expression in macrophages? This would explain and justify the focus on neutrophils for the work presented here.

- Figure 4: the statistics for the same treatment in presence/absence of hsaC5aR should be included in both B and D and discussed in the manuscript. For example is there less neutrophil recuitment to USA300 alone when neutrophils express hsaC5aR?

- Related to the above, in my opinion, it would be very valuable to show in at least one assay that the bacterial infection alone (without further supplementation of purified virulence factors) can clearly affect zebrafish neutrophils function/count in vivo (specifically when they express hsac5aR). For this, the authors could explore later time points than the ones they use in the current Fig. 4 (i.e. to allow a more robust expression of the factors by the bacteria) and/or systemic infection instead of localised infection, where neutrophil counts can be done at the whole animal level. Since some of the c5ar-targeting virulence factors are cytotoxic, the effect of infection +/- hsac5aR on the overall neutrophil count could be a valuable readout.

- Figure 5: Is there any effect on susceptibility, if the larvae are not altered via irf8 morpholino? The effect on survival in WT condition (or ctrMo condition)
would be an important addition to this figure. It would be also valuable to confirm that the observed effects are specific for at least some of the presented virulence factors (i.e. using bacterial mutants or S.a. strains that are naturally negative for the toxins).

Minor comments:
-Figure 1D,E: different groups are labaled with different colors, however some values are close to zero and the groups cannot be easily identified. Could the author clarify this,(i.e. authors could use + and - symbols to identify hsa and dre C5aR expressing cells)?
- L 156. The authors mention that an extensive set of humanised dreC5aR were tested and all failed to achieve toxin-sensitivity. Although negative, these results should be included i.e. in a supplement reporting all the combinations that were tested.

Reviewer 2

Advance summary and potential significance to field

In this study, authors developed a novel humanised zebrafish model. Briefly human neutrophil-specific C5a receptor was transduced in Zebrafish neutrophils. The humanized zebrafish model showed the susceptibility to the S. aureus toxins and reduced neutrophil numbers at the site of infection. Authors reported increased infection-associated mortality in zebrafish. The current manuscript is well written and developed a novel model. This might help study the host-pathogen interaction and therapeutic development.

Comments for the author

Some key points the author should address to improve the current study.

1) What was the C5a receptor transduction efficiency of the Zebrafish?
Zebrafish C5a receptor is known to play an evolutionarily conserved role in cardiac regeneration (PMID: 29348261), in the transgenic Zebrafish did the authors observed phenotypic difference transduced zebrafish?

2) Authors demonstrated that Zebrafish expressing human C5AR1 are more susceptible to S. aureus infection by the survival assay (Fig. 5A, B). The authors should evaluate the functional parameters (inflammatory mediator's expression) in the humanized and non-humanized model to show the difference in the susceptibility to infection. Besides, histological analysis of both the model can provide additional information on the severity of the infection. Authors may refer PMID: 31717750

3) In supplementary (Fig3B), authors showed that in the transgenic model there is no difference in the neutrophil number, authors should consider performing a functional assay to show if there is any difference in the phagocytic or killing potential of neutrophils from both the models. Also, the additional cell type's enumeration like macrophages, etc. in both the model can strengthen the findings that human C5a receptor transduction does not interfere with zebrafish hematopoiesis.

4) Why the authors selected the otic vesicle as an infection site? As multiple anatomical sites can be used for infection in the zebrafish authors should consider the systemic or local infection models to evaluate the variability in human C5a receptor transgenic model.

5) Authors mentioned in the discussion that the model can facilitate the development of vaccines. As in the current study authors selected only one human C5a receptor and host-pathogen interaction is a complex process authors should mention the limitation of this model in studying the host-pathogen interaction or vaccine development strategies.

Reviewer 3

Advance summary and potential significance to field

Buchan and colleagues have made a thorough investigation of S. aureus infection in a humanised zebrafish model and their manuscript is certainly worth to be published in JCS with only minor

clarifications. This work shows the enormous potential of studying human-specific virulence factors of pathogenic bacteria in zebrafish; an approach that will certainly contribute to our future understanding of different bacterial diseases.

Comments for the author

In some cases, I am suggesting a new experiment which in my opinion would strengthen the paper and I leave it to the authors the decision on whether to perform these analyses or leave the text as it is.

- I would remove from the abstract on line 42 that the increased associated mortality is a direct result of the interaction between S. aureus and the receptor. If the authors want to keep such a strong statement, more evidence should be provided.

- In Figure 1, the authors look at the binding of FITC-labelled C5a to the C5aR. Since the analysis has been done with flow cytometry, I wonder if they used tripsin for detaching cells. In case the authors did use tripsin, could its proteolytic activity have influenced the results? Please comment.

- In figure 1D and E, Dapi is used to assess permeability of cells in presence of S.aureus toxins PVL and HlgCB. The differences between groups are striking and therefore there are virtually no doubts that indeed the toxins increase the permeability in U937-hsaC5aR cells. Regardless, in my view, permeability should be analyzed with a non-permeable molecule such as propidium iodide for more reliable results.

Also, since these toxins are supposed to cause cell lysis, I wonder if the authors had a look at cell death in presence of PVL and HlgCB.

- The authors show, in figure 1C that U937-hsaC5aR cells display high C5aR activity in presence of hsaC5a and in absence of the toxin CHIPS while conversely there is nearly no activity in presence of CHIPS. I wonder why the authors did not have a look at the in vivo effects of CHIPS for leucocyte recruitment.

- The authors observe that MRSA strain USA300 causes similar recruitment of neutrophils in the otic vesicle for zebrafish C5AR1 positive or negative while the co-injection of the bacterium with either of the two toxins is instead able to reduce the number of neutrophils at the infection site only in C5AR1 positive zebrafish. Have the authors checked if this strain of S.aureus is producing these toxins in vivo?

The authors conclude that the reduction of neutrophils seen for HlgCB is likely due to the pore forming activity of HlgCG. Have the authors checked at overall numbers of neutrophils or markers of cell death in the embryo to corroborate their analysis?

- In figure 5 the susceptibility of zebrafish larvae (possessing mainly neutrophils after irf8 morpholino injection) to two doses of S.aureus is analyzed. This study, especially for 2000 injected CFU, gives a clear indication that C5AR1-positive zebrafish are more susceptible to the infection than the C5AR1-negavie ones. The authors cite the paper of Colucci-Guyon et al. 2011 JCS where evidence is provided for neutrophils being very efficient in taking up surface associated but not fluid borne bacteria. For surface associated bacteria, Colucci-Guyon and colleagues injected E.coli subcutaneously or in the otic vescicle, where they observe neutrophils readily take up the bacteria. Personally I think that analyzing the susceptibility to S.aureus when the bacterium is injected subcutaneously or in the otic vescicle could be a good alternative to injecting intravenously zebrafish embryos lacking (only temporarily) macrophages.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

Buchan et al. engineer a humanised zebrafish line, where neutrophils express the human c5a receptor, a target of an array of S. aureus host-specific virulence factors. Accordingly, they demonstrate that hC5aR expression by zebrafish neutrophils leads to increased susceptibility of the larvae to staphylococcal infection in irf8-KD condition.

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The manuscript is well-written and structured and represents a valuable advance in the field. However, I have a few suggestions for improvement, as I outline below.

Reviewer 1 Comments for the Author:

- **Figure 2: membrane-bound expression of hsac5aR-clover: the figure 2C is not very clear. Perhaps a higher resolution image could confirm this more robustly. Also, in the bottom-right part of the image, it seems that the cytosolic mcherry is outside the cell, as it would be outlined by clover. Probably this is due to the live imaging. However, if the cell is moving, this might also compromise the conclusions made on mcherry/clover not overlapping across the yellow line above.**

We thank the reviewer for this comment. The timelapse movie from which the snapshot in Figure 2C was taken more clearly shows that the clover signal appears at the perimeter of the neutrophil, and we have now added this movie as Supplementary Movie 1.

- **Figure 3B: Why does expression of hsaC5aR lead to neutrophils that are less capable to respond to dreC5a (which they should still sense via the endogenous dreC5aR)? The authors should comment on this. Also, it would be useful to include statistical information for groups injected with the same chemoattractant but expressing/non-expressing hsac5aR. This would also justify why the authors use two-way ANOVA, rather than one-way ANOVA.**

We agree with the observation by the reviewer. We have now included the statistical comparisons between groups injected with the same chemoattractant as suggested. A two-way ANOVA shows that humanised fish injected with dreC5a indeed recruit significantly fewer neutrophils than non-humanised fish (**, $p = 0.0032$). Although we have not investigated this in detail, our interpretation of this finding is that the addition of the human C5a receptor to the neutrophil surface slightly impairs endogenous signalling due to competition between the two receptors. To prevent any inherent differences between the two fish lines from affecting the neutrophil migration assays presented later in the manuscript, we typically compared neutrophil migration in the absence and presence of the toxins within each line, as opposed to between the two lines. Furthermore, the absence of a difference in neutrophil numbers between the two fish lines upon *S. aureus* USA300 infection alone, which is now more clearly indicated in Figure 4 based on the reviewer's suggestions, shows that the difference in response to dreC5a does not play a role in the infection context. We have also included the comparison between non- humanised and humanised fish injected with hsaC5a (****, $p < 0.0001$), which supports our conclusion that zebrafish neutrophils expressing hsaC5aR become able to respond to gradients of injected hsaC5a *in vivo*.

- **Related to the above, could the authors show, or refer to studies, reporting that hsac5aR/dreC5aR is expressed by human/zebrafish neutrophils, and how for example this differs in levels from expression in macrophages? This would explain and justify the focus on neutrophils for the work presented here.**

In addition to a wide variety of tissues and cell types, it is known that C5AR1 is highly expressed by macrophages and neutrophils isolated from both murine and human blood (Laumonnier et al., 2017). Our focus on neutrophils stems from the finding that neutrophils are more susceptible to targeting and lysis by Panton-Valentine Leukocidin (PVL) than macrophages (Meyer et al. 2009 *Infection and Immunity*). Furthermore, C5AR1 was found to be the primary target of PVL, and human neutrophils were shown to express roughly tenfold higher levels of C5AR1 compared with human monocytes (Spaan et al. 2013 *Cell Host & Microbe*). As our focus was to mimic human infections by investigating the interaction between human- specific pore-forming leukotoxins and human C5AR1 using the

zebrafish, we chose to express the receptor in zebrafish neutrophils. We have now included additional discussion of this point on page 13 line 266-274.

While the complement system has been studied extensively in humans, knowledge of the zebrafish complement system is less complete. The only information available is that zebrafish possess a C5AR1 orthologue and that the zebrafish C5AR1 is upregulated during cardiac regeneration (Natarajan et al. 2018 *Circulation*). To our knowledge, our work is the first time the C5a-C5aR signalling axis has been shown to mediate neutrophil migration in zebrafish. While outside the scope of the current study, we agree with the reviewer that it will be of great interest to characterize the C5a-C5aR signalling pathway, as well as the complement system as a whole in zebrafish, and to determine whether C5aR expression in neutrophils and macrophages is similar to the human situation.

- **Figure 4: the statistics for the same treatment in presence/absence of hsaC5aR should be included in both B and D and discussed in the manuscript. For example, is there less neutrophil recruitment to USA300 alone when neutrophils express hsaC5aR?**

We thank the reviewer for this comment and have added the requested statistics to the figure, and some material to the discussion. The additional statistics provided are as follows:

Figure 4 B, humanised compared to non-humanised:

USA300	Non-significant	$p > 0.9999$
USA300 + PVL	**	$p = 0.0015$

Figure 4 D, humanised compared to non-humanised:

USA300	Non-significant	$p > 0.9999$
USA300 + HlgC	Non-significant	$p = 0.126$
USA300 +	**	$p = 0.0039$

For both B) and D) there is no significant difference in neutrophil recruitment in fish infected with USA300 alone, indicating that no inherent migration defect exists between the non-humanised and humanised fish lines under these conditions. In both cases, there is a significant decrease in neutrophil numbers in the humanised fish compared to the non-humanised group when injected with USA300 and the whole toxin (PVL/HlgCB), further corroborating our conclusion that targeting of C5AR1 in zebrafish neutrophils by the *S. aureus* toxins interferes with neutrophil recruitment. It is also worth noting in Figure 4D that fish injected with only the HlgC portion of the HlgCB toxin show no significant difference in neutrophil numbers between non-humanised and humanised groups, suggesting that the whole toxin is required for the reduction in neutrophils and supporting our notion that this effect is likely mediated by pore-formation and cell death rather than C5aR inhibition by HlgC alone.

- **Related to the above, in my opinion, it would be very valuable to show in at least one assay that the bacterial infection alone (without further supplementation of purified virulence factors) can clearly affect zebrafish neutrophils function/count in vivo (specifically when they express hsac5aR). For this, the authors could explore later time points than the ones they use in the current Fig. 4 (i.e. to allow a more robust expression of the factors by the bacteria) and/or systemic infection instead of localised infection, where neutrophil counts can be done at the whole animal level. Since some of the c5ar-targeting virulence factors are cytotoxic, the effect of infection +/- hsac5aR on the overall neutrophil count could be a valuable readout.**
We thank the reviewer for this comment. We agree that this would be an interesting addition and indeed have performed extensive experiments attempting to address this specific point. In a systemic infection model, protection against infection is mediated largely by macrophages (Colucci-Guyon et al., 2011; Prajsnar et al., 2012) and we believe this is likely to explain the lack of mortality signal. We have informally assessed neutrophil number in this assay and not identified a noticeable change in neutrophil number. This may be due to the balancing of neutrophil numbers by emergency granulopoeisis. We believe the correct experiment is the irf8 MO experiment (Figure 5), which forces the model to be dependent on neutrophils for host defence. For this reason, we did not include the systemic model data in the manuscript.

- **Figure 5: Is there any effect on susceptibility, if the larvae are not altered via irf8 morpholino? The effect on survival in WT condition (or ctrMo condition) would be an important**

addition to this figure. It would be also valuable to confirm that the observed effects are specific for at least some of the presented virulence factors (i.e. using bacterial mutants or S.a. strains that are naturally negative for the toxins).

We appreciate this question by the reviewer, and have added survival of non-morphant fish to the manuscript as Supplementary Figure 7.. As discussed above, we found that wild-type hC5aR-negative fish are equally as susceptible to infection as hC5aR-positive fish and believe that this is due to the relatively minor role of neutrophils during infection in this systemic infection model (Colucci-Guyon et al., 2011; Prajsnar et al., 2012). As the reviewer points out earlier, macrophages also express C5aR, but our chosen transgene limits the models we can use to address the role of C5aR in *S. aureus* host- targeting. It is worth noting that these experiments have not been performed in murine systems – underscoring the novelty and complexity of the experiments we show in this manuscript.

Minor comments:
-Figure 1D,E: different groups are labelled with different colors, however some values are close to zero and the groups cannot be easily identified. Could the author clarify this,(i.e. authors could use + and - symbols to identify hsa and dre C5aR expressing cells)?
We agree with the reviewer and have now more clearly marked the different groups by adding a blue and red background shading to the graphs.

-L 156. The authors mention that an extensive set of humanised dreC5aR were tested and all failed to achieve toxin- sensitivity. Although negative, these results should be included i.e. in a supplement reporting all the combinations that were tested.
As requested by the reviewer, an overview of the tested variants has now been incorporated in the revised manuscript as Supplementary Figure 3.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this study, authors developed a novel humanised zebrafish model. Briefly, human neutrophil-specific C5a receptor was transduced in Zebrafish neutrophils. The humanized zebrafish model showed the susceptibility to the S. aureus toxins and reduced neutrophil numbers at the site of infection. Authors reported increased infection-associated mortality in zebrafish. The current manuscript is well written and developed a novel model. This might help study the host-pathogen interaction and therapeutic development.

Reviewer 2 Comments for the Author:
Some key points the author should address to improve the current study.

1) What was the C5a receptor transduction efficiency of the Zebrafish?
Zebrafish C5a receptor is known to play an evolutionarily conserved role in cardiac regeneration (PMID: 29348261), in the transgenic Zebrafish did the authors observed phenotypic difference transduced zebrafish?

The creation of the lyz:hsaC5AR1-Clover line was a difficult process because of low transduction efficiency (around 2% of injected fish expressed the transgene). However, after raising the transgenic fish, we identified a founder after only breeding around 4 pairs out of 30 possible founders.

Although we did not investigate cardiac development or heart function, this is a very interesting question. In terms of general health, we observed no phenotypic differences between our wild-type strain and the transgenic strain. The impact of transgene expression on overall physiology may have been alleviated by the restricted expression of the hC5aR transgene confined to neutrophils. Furthermore, the data presented in Figure 3 suggests that the endogenous dreC5aR remains functional in the transgenic animals.

2) Authors demonstrated that Zebrafish expressing human C5AR1 are more susceptible to S. aureus infection by the survival assay (Fig. 5A, B). The authors should evaluate the functional parameters (inflammatory mediator's expression) in the humanized and non-humanized model to show the difference in the susceptibility to infection. Besides, histological analysis of both the model can provide additional information on the severity of the infection. Authors may refer PMID: 3171775

We thank the reviewer for these suggestions. As the reviewer suggests, we have examined the most important functional parameter - neutrophil recruitment to sites of inflammation in hsaC5AR1-positive zebrafish using a tail transection assay. These data, which have now been added to Supplementary figure 5, shows that neutrophils are recruited to tail wounds in modestly lower numbers compared to wild-type fish. We anticipate that this is due to disruption of normal chemotaxis by crosstalk with the endogenous zebrafish receptor. It would be intriguing to examine how, as suggested, this has affected the expression/production of important inflammatory mediators e.g. IL-1β, TNF-α etc, in order to determine why this effect occurs, but we feel this is beyond the scope of the current manuscript.

3) In supplementary (Fig3B), authors showed that in the transgenic model there is no difference in the neutrophil number, authors should consider performing a functional assay to show if there is any difference in the phagocytic or killing potential of neutrophils from both the models. Also, the additional cell type's enumeration like macrophages, etc. in both the model can strengthen the findings that human C5a receptor transduction does not interfere with zebrafish hematopoiesis.

We thank the reviewer for this suggestion. We have now performed somite infection assays by injecting 1,000 CFU of USA300 stained with Alexafluor-647 into non-humanised (lyz:nfsB-mCherry) and humanised (lyz:nfsB-mCherry; lyz:hsaC5AR1-Clover) larvae (Supplementary Figure 6). We then imaged migration of the neutrophils to the injection site and analysed their velocity, distance migrated, meandering index (i.e. a measure of the 'straightness' of migration) and displacement (the neutrophil's overall change in position). This showed that in humanised neutrophils these parameters are modestly lower in all measurements with the exception of meandering index. This is in line with our finding that these neutrophils are disrupted in their recruitment to sites of inflammation (Supplementary Figure 5).

Despite the modest defect in neutrophil recruitment in this somite infection model, we find no difference in overall recruitment between non-humanised and humanised fish to sites of infection at the timepoint studied (4 hours post infection) in our otic vesicle infection assays (Fig. 3 & 4). Our suggestion is that although initial recruitment is impaired compared with non-humanised larvae, it does not result in any meaningful susceptibility over time, as demonstrated in our otic vesicle assays. Additionally, other studies that involved ectopic expression of human proteins (Buchan et al., 2019) or other GPCRs such as cxcr1 and cxcr2 (Coombs et al., 2019) demonstrated no impact on neutrophil haematopoiesis, suggesting that any disruption of this process is unlikely. However, it is true that we cannot rule out the idea that if there is disruption to the immune response, that this observation is contributing to the observation.

4) Why the authors selected the otic vesicle as an infection site? As multiple anatomical sites can be used for infection in the zebrafish, authors should consider the systemic or local infection models to evaluate the variability in human C5a receptor transgenic model.

At the beginning of our study, we compared several infection models including the somite and otic vesicle infection sites. From this we concluded that the otic vesicle model was most compatible with our experimental setup, and allowed the analysis of relatively large groups of larvae simultaneously (8 groups of 20 larvae per experiment). Moreover, the otic vesicle is a defined structure that typically does not contain circulating neutrophils, minimising the possibility of false-positives. In contrast, the somite infection model did not allow proper evaluation of neutrophil recruitment due to the lack of definition of the somite muscle tissue. Furthermore, its proximity to the caudal haematopoietic tissue introduced counting errors due to the inability to distinguish between neutrophils that were migrating to the site of infection, and neutrophils that were naturally circulating throughout the area near the caudal haematopoietic tissue.

We also considered using the systemic model, however the predominant phagocytes involved in the systemic response to infection in the zebrafish model are macrophages, while neutrophils associate mostly with surface-associated microbes (Colucci-Guyon et al., 2011; Prajsnar et al., 2012), and so the systemic model would be unlikely to show much of a difference as our transgene is neutrophil-specific. We demonstrate this in determining the survival of hC5aR-positive vs hC5aR-negative fish after *irf8* knockdown (Figure 5).

5) Authors mentioned in the discussion that the model can facilitate the development of vaccines. As in the current study authors selected only one human C5a receptor and host-pathogen interaction is a complex process, authors should mention the limitation of this model in studying the host-pathogen interaction or vaccine development strategies.

We agree with the reviewer that the current model is a promising starting point for a more comprehensive zebrafish infection model that would ideally incorporate many host-pathogen interactions. Here, we performed a proof-of-principle study to show that humanisation of zebrafish components provides a promising approach in mimicking human-specific host-pathogen interactions in the zebrafish, and provides a useful platform for validating major virulence factors (PVL/HlgCB) of *S. aureus*, which has until now not been possible in other models. Future studies are required to refine this model by applying our detailed knowledge of the interaction between the many *S. aureus* virulence factors and the human host for the targeted humanisation of additional fish components. Previous studies have shown that accurate representation of host-pathogen interactions is important for vaccine studies (Salgado-Pabón and Schlievert, 2014).

We have added a brief discussion of the primary limitation of the zebrafish as a model for host-pathogen interactions to the discussion, as well as highlight that future studies should fully characterise the complement system in zebrafish. This step would considerably accelerate the study of interactions between pathogens and the zebrafish innate immunity.

Reviewer 3 Advance Summary and Potential Significance to Field:
Buchan and colleagues have made a thorough investigation of S. aureus infection in a humanised zebrafish model and their manuscript is certainly worth to be published in JCS with only minor clarifications. This work shows the enormous potential of studying human-specific virulence factors of pathogenic bacteria in zebrafish; an approach that will certainly contribute to our future understanding of different bacterial diseases.

Reviewer 3 Comments for the Author:
In some cases, I am suggesting a new experiment which in my opinion would strengthen the paper and I leave it to the authors the decision on whether to perform these analyses or leave the text as it is.

- **I would remove from the abstract on line 42 that the increased associated mortality is a direct result of the interaction between S. aureus and the receptor. If the authors want to keep such a strong statement, more evidence should be provided.**

We thank the reviewer for the suggestion and we have removed this statement from the abstract.

- **In Figure 1, the authors look at the binding of FITC-labelled C5a to the C5aR. Since the analysis has been done with flow cytometry, I wonder if they used tripsin for detaching cells. In case the authors did use tripsin, could its proteolytic activity have influenced the results? Please comment.**

This is a good point raised by the reviewer. However, since these U937 cells grow in suspension, trypsin was not required to detach the cells and will therefore not affect the results.

- **In figure 1D and E, Dapi is used to assess permeability of cells in presence of S.aureus toxins PVL and HlgCB. The differences between groups are striking and therefore there are virtually no doubts that indeed the toxins increase the permeability in U937-hsaC5aR cells. Regardless, in my view, permeability should be analyzed with a non-permeable molecule such as propidium iodide for more reliable results. Also, since these toxins are supposed to cause cell lysis, I wonder if the authors had a look at cell death in presence of PVL and HlgCB.**

These are excellent suggestions by the reviewers. However we have not analysed cell death independently from the DAPI- based flow cytometry assay. While we have only performed these specific assays for the current manuscript with DAPI, we have used DAPI and propidium iodide interchangeably in the past in similar assays without any major differences in experimental outcome (Spaan et al., 2013, *Cell Host & Microbe*).

- **The authors show, in figure 1C that U937-hsaC5aR cells display high C5aR activity in presence of hsaC5a and in absence of the toxin CHIPS while conversely there is nearly no activity in presence of CHIPS. I wonder why the authors did not have a look at the in vivo effects of CHIPS for leucocyte recruitment.**

In this study, we have generated a humanised, hC5aR knock-in zebrafish model. While this gain-of-function approach allows studying the effects of the *S. aureus* toxins, it unfortunately does not allow the study of receptor-inhibitory virulence factors, as even though the exogenous hC5aR receptor is expected to be inhibited by CHIPS, the endogenous drC5a-drC5aR signalling remains unaffected. However this is a very interesting point and based on the successful application of this model in this study, we do envision future studies to expand this and replace the endogenous drC5aR entirely with the hC5aR gene to allow more comprehensive studies towards the interaction between additional S. aureus virulence factors, including CHIPS, and the (human) host.

- **The authors observe that MRSA strain USA300 causes similar recruitment of neutrophils in the otic vesicle for zebrafish C5AR1 positive or negative while the co-injection of the bacterium with either of the two toxins is instead able to reduce the number of neutrophils at the infection site only in C5AR1 positive zebrafish. Have the authors checked if this strain of S.aureus is producing these toxins in vivo?**

Although we did not directly study expression and production of PVL and γ-Haemolysin CB in this work, both are major virulence factors secreted by the USA300 LAC strain used in this study and are known to be expressed and produced in broth culture, mostly between mid-log and early stationary phase (Spaan et al. 2014). PVL and HlgCB are also expressed under various broth culture conditions in other *S. aureus* strains (Bronner et al. 2000 *Appl Environ Micro*).

The authors conclude that the reduction of neutrophils seen for HlgCB is likely due to the pore forming activity of HlgCG. Have the authors checked at overall numbers of neutrophils or markers of cell death in the embryo to corroborate their analysis?
In Supplemental Figure 4, we show that overall neutrophil numbers between humanised and non-humanised fish are equal, so a difference in neutrophil numbers as a source of variation is unlikely. However, we did not enumerate the total numbers of neutrophils between the non-humanised and humanised fish following toxin treatment. Unfortunately, staining for markers of cell death was incompatible with the otic vesicle injection model.

- **In figure 5 the susceptibility of zebrafish larvae (possessing mainly neutrophils after irf8 morpholino injection) to two doses of S.aureus is analyzed. This study, especially for 2000 injected CFU, gives a clear indication that C5AR1-positive zebrafish are more susceptible to the infection than the C5AR1-negavie ones. The authors cite the paper of Colucci- Guyon et al. 2011 JCS where evidence is provided for neutrophils being very efficient in taking up surface associated but not fluid borne bacteria. For surface associated bacteria, Colucci-Guyon and colleagues injected E.coli subcutaneously or in the otic vescicle, where they observe neutrophils readily take up the bacteria. Personally I think that analyzing the susceptibility to S.aureus when the bacterium is injected subcutaneously or in the otic vescicle could be a good alternative to injecting intravenously zebrafish embryos lacking (only temporarily) macrophages.**

This is a great suggestion by the reviewer. We initially compared several infection models including the somite model (as it is more neutrophil-dependent) to study zebrafish susceptibility to *S. aureus* infection. However, these initial somite injection survival experiments required very high doses of *S. aureus* (between 6,000 and 8,000 CFU), suggesting that this infection route is very resistant to inducing mortality in larvae. We therefore decided to use the otic vesicle route as we were concerned we would need to inject such high CFUs into the somites that it would make the infection physiologically unrepresentative.

Second decision letter

MS ID#: JOCES/2020/252205

MS TITLE: Human-specific staphylococcal virulence factors enhance pathogenicity in a humanised zebrafish C5a receptor model.

AUTHORS: Kyle D Buchan, Michiel van Gent, Tomasz K Prajsnar, Nikolay V Ogryzko, Nienke WM de Jong, Julia Kolata, Simon J Foster, Jos AG Van Strijp, and Stephen A Renshaw
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The reviewers improved the manuscript and addressed my points and those raised by the other reviewers. This work represents a great advance in the field and I look forward to seeing it published.

Comments for the author

The reviewers improved the manuscript and addressed my points and those raised by the other reviewers. This work represents a great advance in the field and I look forward to seeing it published.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all of my prior concerns.

Comments for the author

The authors have addressed all of my prior concerns.

Reviewer 3

Advance summary and potential significance to field

-/-

Comments for the author

I am satisfied with the answers provided by the authors.