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Exploiting dominant-negative toxins to combat Staphylococcus aureus pathogenesis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 August 2015

We have already received the enclosed three reports on your EMBO reports submission. As you will see, all the referees are highly supportive of the study.

Referees 1 and 3, which are toxin experts, are largely satisfied with the study and referee 3 only raises a couple of minor issues, which can be easily addressed. Referee 2 is an expert on S. aureus pathogenesis and requests some strengthening of the infectivity data. I mostly agree with him/her that providing more data on this aspect will undoubtedly raise the importance of the work. In this regard, testing the efficacy of the DN toxin in a sepsis model, assessing whether it can antagonize LukAB and whether it prevents bacterial-induced PMN cytotoxicity would be important. The requested macrophage analysis would of course be nice, but I would agree it goes beyond the scope of this study if you chose not to pursue it.

If there are any questions regarding the extent of revision, please contact me. I will be in the office until the end of the week. Thereafter, Martina Rmbold will be the handling editor for your study.

REFEREE REPORTS

Referee #1:

This is an excellent study, which has clear implications for development of a new class of therapeutics against Staphylococcus aureus infections. The authors have demonstrated dominant negative properties of certain mutated forms of bicomponent pore forming toxins produced by this

organism. These mutated forms lack the ability to form a transmembrane beta barrel, but are able to co-oligomerize with wild-type complementary subunits, yielding inactive complexes. The results reported raise many intriguing questions, which the authors will undoubtedly address in future publications.

Referee #2:

The authors show that four of the S. aureus bi-component toxins share a common glycine rich motif that when inactivated exerts dominant-negative effects to native as well as heterologous subunits. The dominant negative proteins prevent pore formation by the native proteins and can prevent toxicity by other leukocidins that share the same binding receptors on neutrophils. This is an interesting study and holds potential for future staphylococcal therapeutics. Some additional data noted below would further strengthen the study, in particular in vivo experiments.

The ability of the dominant-negative toxins to prevent toxicity in vivo is primarily tested using purified protein. Only one experiment is shown at 96h post infection with dominant negative protein and live organisms. The authors have published that lukED mutants have reduced virulence in a sepsis mortality model. Administration of the dominant-negative toxin in the sepsis model would be an important confirmation that such mutant proteins potentially represent a novel biologic.

The four bi-component toxins examined have fairly conserved amino acid identity. The other leukocidin LukAB does not. The authors do raise the point that the glycine domain does exist in LukAB. Even though LukAB is dissimilar to the other toxins, constructing a mutation in its glycine domain would seem prudent to test if it can antagonize the activity of LukAB. Even if it does not it would be important data to include in the study.

The blocking activity of the dominant negative toxin was tested in vitro with purified protein and culture supernatants. Can they protect against bacterial-induced cytotoxicity in PMNs?

The authors focused on neutrophils. One or two key experiments showing that this is true for macrophages as well would further strengthen the conclusions.

The in vivo toxicity experiments do mention numbers of mice but it is unclear if these are from a single experiment or collated data from multiple experiments. If a single experiment they should be repeated. This applies to most of the figures, which don't mention numbers of times the experiments were repeated.

Referee #3:

The manuscript "Exploiting dominant-negative toxins to combat Staphylococcus aureus Pathogenesis" by Reyes-Robles et al. explores the ability of recombinant mutant Leukocidin subunits to inhibit pore formation and cell-killing activity in vitro and in vivo. Specifically, the mutation involves deletion of a Gly-rich motif found in the pore-forming beta-hairpin that comprises the membrane spanning beta-barrel. Leukocidins are heterodimeric toxins (tetramer of dimers), which pair in order to make an octameric complex. It is found in cell-viability assays that this deletion made the Gly-rich deletion mutant dominant negative. The mixture of this mutant and its wild type counterpart afforded protection of human PMN cells. Most of the dominant-negative mutants protected mice in toxin challenge assays. One pair, LukED, was tested in an infection model, and it showed a 1.5 to 2 log reduction of the infection in the liver.

I have no major concerns with this manuscript. My comments if addressed would only improve the overall clarity of the manuscript.

Throughout the results I wanted an indication of the stoichiometry required to observe the dominant negative phenotype. The Discussion mentions 1:2 stoichiometry, but nowhere in the text or figures did I encounter the evidence for this statement of 1:2. I would expect for a dominant negative phenotype in a heterodimeric octamer that 1:4 would be the optimal value. I think the authors should briefly comment on this and make the stoichiometry more clear throughout the results.

The crystal structure figures were a bit confusing in Fig. 1C. The legend says on line 763-64 that the top and side views are of the pore, but to me the figure of the top view is really the pre-pore. At the bottom of the legend (lines 767-78) the text says pre-pore. This needs to be clarified. The colors in Fig. 1C are too muted to distinguish the subunits. The blues and grays are too close together (especially in the shaded areas of the "Top view"), and the red and purple are too close in color. This may be my printer or monitor. I would add a cyan or gold color to give more contrast.

Minor edits

Line 28: Change "in" to "on".
Line 64: Change "disease" to plural.

1st Revision - authors' response 04 December 2015

We appreciate the time and thoroughness exhibited by the referees and the editor. We have taken much care and effort to address each comment. Below is a point-by-point response to the reviewers' comments.

Referee #1: *This is an excellent study, which has clear implications for development of a new class of therapeutics against Staphylococcus aureus infections. The authors have demonstrated dominant negative properties of certain mutated forms of bicomponent pore forming toxins produced by this organism. These mutated forms lack the ability to form a transmembrane beta barrel, but are able to co-oligomerize with wild-type complementary subunits, yielding inactive complexes. The results reported raise many intriguing questions, which the authors will undoubtedly address in future publications.*

Response: We appreciate that this referee found our study important.

Referee #2: *The authors show that four of the S. aureus bi-component toxins share a common glycine rich motif that when inactivated exerts dominant-negative effects to native as well as heterologous subunits. The dominant negative proteins prevent pore formation by the native proteins and can prevent toxicity by other leukocidins that share the same binding receptors on neutrophils. This is an interesting study and holds potential for future staphylococcal therapeutics. Some additional data noted below would further strengthen the study, in particular in vivo experiments.*

The ability of the dominant-negative toxins to prevent toxicity in vivo is primarily tested using purified protein. Only one experiment is shown at 96h post infection with dominant negative protein and live organisms. The authors have published that lukED mutants have reduced virulence in a sepsis mortality model. Administration of the dominant-negative toxin in the sepsis model would be an important confirmation that such mutant proteins potentially represent a novel biologic.

Response: We have now tested the dominant-negative mutants in several permutations of our lethal model of bloodstream infection and have observed no protection. Subsequent experiments using the *in vivo* intoxication model described in our study reveal that the dominant negative toxins exhibit short half-life when injected into the mouse. Thus, additional work is needed to improve the bioavailability of these proteins *in vivo*. Nevertheless, our *in vivo* intoxications and bacterial burden data support the notion that these mutant toxins could be further developed into novel anti-staphylococcal agents.

The four bi-component toxins examined have fairly conserved amino acid identity. The other leukocidin LukAB does not. The authors do raise the point that the glycine domain does exist in LukAB. Even though LukAB is dissimilar to the other toxins, constructing a mutation in its glycine domain would seem prudent to test if it can antagonize the activity of LukAB. Even if it does not it would be important data to include in the study.

Response: We thank the reviewer for this excellent suggestion. Since LukAB is more stable when purified as a dimer, we have now generated the corresponding mutations in LukA and LukB

together and found that they were indeed protective against LukAB produced by WT *S. aureus* in the context of human PMN infection *ex vivo*. The corresponding data has now been included in a revised version (Figure 4). Unfortunately, while the mutated proteins were produced at WT levels by *S. aureus*, we were unable to purify them, as the proteins precipitated during the dialysis process.

The blocking activity of the dominant negative toxin was tested in vitro with purified protein and culture supernatants. Can they protect against bacterial-induced cytotoxicity in PMNs?

Response: The main problem in executing the experiments suggested by the referee is that during *ex vivo* infection with WT *S. aureus*, LukAB is the toxin responsible for lysis of human PMNs. We tried using strains lacking *lukAB,* but were unable to measure any cell lysis in infections with MOI of 100 lasting up to four hours.

We have now developed an assay where human PMNs are infected with a mutant *S. aureus* strain mutated for all the leukocidin, but engineered to overproduce HlgCB or PVL. We found that in the absence of LukAB, these two toxins are the most active (these data are now included in the revised manuscript). Our new data demonstrate that the dominant-negative toxins can indeed protect against infection (see Figure 3A-D).

The authors focused on neutrophils. One or two key experiments showing that this is true for macrophages as well would further strengthen the conclusions.

Response: This is a good idea, however, we believe testing the dominant-negative toxins on macrophages falls beyond the scope of this manuscript, therefore was not included in this submission. We have generated preliminary data with red blood cells suggesting that the mutated toxins also exhibit a dominant negative phenotype in this setup.

The in vivo toxicity experiments do mention numbers of mice but it is unclear if these are from a single experiment or collated data from multiple experiments. If a single experiment they should be repeated. This applies to most of the figures, which don't mention numbers of times the experiments were repeated.

Response: We thank the reviewer for the helpful comment. We have now included the required information on each figure. Each *in vivo* experiment was at least performed twice with the indicated number of mice per each experiment.

Referee #3:
The manuscript "Exploiting dominant-negative toxins to combat Staphylococcus aureus Pathogenesis" by Reyes-Robles et al. explores the ability of recombinant mutant Leukocidin subunits to inhibit pore formation and cell-killing activity in vitro and in vivo. Specifically, the mutation involves deletion of a Gly-rich motif found in the pore-forming beta-hairpin that comprises the membrane spanning beta-barrel. Leukocidins are heterodimeric toxins (tetramer of dimers), which pair in order to make an octameric complex. It is found in cell-viability assays that this deletion made the Gly-rich deletion mutant dominant negative. The mixture of this mutant and its wild type counterpart afforded protection of human PMN cells. Most of the dominant-negative mutants protected mice in toxin challenge assays. One pair, LukED, was tested in an infection model, and it showed a 1.5 to 2 log reduction of the infection in the liver.

I have no major concerns with this manuscript. My comments if addressed would only improve the overall clarity of the manuscript.

Throughout the results I wanted an indication of the stoichiometry required to observe the dominant negative phenotype. The Discussion mentions 1:2 stoichiometry, but nowhere in the text or figures did I encounter the evidence for this statement of 1:2. I would expect for a dominant negative phenotype in a heterodimeric octamer that 1:4 would be the optimal value. I think the authors should briefly comment on this and make the stoichiometry more clear throughout the results.

Response: We have deduced the stoichiometry based on the data presented in Figure 3 comparing the concentration of the WT toxins to the concentration of the domain negative needed to block the toxin. Based on these data we predicted ratio of 1:1 to 1:2 of WT to mutant across all the toxins (see Figure 3). Similar data was obtained when using and LD50 of WT LukED (data nor shown).

The crystal structure figures were a bit confusing in Fig. 1C. The legend says on line 763-64 that the top and side views are of the pore, but to me the figure of the top view is really the pre-pore. At the bottom of the legend (lines 767-78) the text says pre-pore. This needs to be clarified. The colors in Fig. 1C are too muted to distinguish the subunits. The blues and grays are too close together (especially in the shaded areas of the "Top view"), and the red and purple are too close in color. This may be my printer or monitor. I would add a cyan or gold color to give more contrast.

Response: We thank the reviewer for this suggestion. The appropriate changes have been made to the figure, both the contrasting colors of the structures and the figure legend.

Minor edits
Line 28: Change "in" to "on". This is now fixed.
Line 64: Change "disease" to plural. This is now fixed.

2nd Editorial Decision 21 December 2015

Thank you for the submission of your research manuscript to our journal. We have now received the report of the referee that was asked to assess it (copied below).

As you will see, the referee supports publication of the manuscript but requests to incorporate the information regarding the half-life of the anti-toxins and the attempt to test protection of the dominant-negative proteins in a lethal model of bloodstream infection into the manuscript. This caveat needs to be discussed in the paper.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS
Referee #2:

This resubmission "Exploiting dominant-negative toxins to combat Staphylococcus aureus pathogenesis" attempts to address the major concern the authors did not adequately demonstrate the ability of the dominant-negative proteins to protect against infection. The attempt to address this issue with new data was not successful, as the authors suggest that the half-lives of the proteins are too short to provide a sustained in vivo effect. While disappointing, this does not negate the overall impact of the work using a novel "anti-toxin" approach to ameliorate the pathology of virulent S. aureus infection.

This information regarding the half-life and attempts at protection should be incorporated into the manuscript. All the other concerns were either fully addressed or efforts were made to address them.

2nd Revision - authors' response 30 December 2015

Below is a point-by-point response to the reviewer's and editor's remaining comments.

Editor:
1. As you will see, the referee supports publication of the manuscript but requests to incorporate the information regarding the half-life of the antitoxins and the attempt to test protection of the dominant-negative proteins in a lethal model of bloodstream infection into the manuscript. This caveat needs to be discussed in the paper.

Response: We have included these negative data (Fig EV2) and have added text describing these findings to the manuscript (Page 12; lines #280-292).

Referee #2:
This resubmission "Exploiting dominant-negative toxins to combat Staphylococcus aureus

pathogenesis" attempts to address the major concern the authors did not adequately demonstrate the ability of the dominant-negative proteins to protect against infection. The attempt to address this issue with new data was not successful, as the authors suggest that the half-lives of the proteins are too short to provide a sustained in vivo effect. While disappointing, this does not negate the overall impact of the work using a novel "anti-toxin" approach to ameliorate the pathology of virulent S. aureus infection. This information regarding the half-life and attempts at protection should be incorporated into the manuscript. All the other concerns were either fully addressed or efforts were made to address them.

Response: We have included these negative data (Fig EV2) and have added text describing these findings to the manuscript (Page 12; lines #280-292).

3rd Editorial Decision 05 January 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.