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Staphylococcus aureus Staphopain A inhibits CXCR2 dependent neutrophil activation and chemotaxis

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1st Editorial Decision 30 August 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees, whose comments are enclosed below. As you will see, all the reviewers do recognise the potential interest in your identification of CXCR2 as a substrate for Staphopain A. However, all also raise major concerns with the study that - in our assessment - would preclude publication of your work in the EMBO Journal at this point.

Their reports are explicit, but the over-riding concern is the relevance of the identified mechanism in the infection context. While we do recognise that in vivo experiments may not be feasible, it is clear that your study does not currently go far enough to demonstrate that Staphopain A mediated CXCR2 cleavage is patho-physiologically important, or to demonstrate conclusively that this event is responsible for the effects of Staphopain A on neutrophil functions.

Given these clear concerns, I am afraid that we can not offer to publish your manuscript. With the high number of submissions we receive, we can only consider those which receive an enthusiastic report from at least a majority of the referees upon initial review. I am sorry we can not be more positive on this occasion, but we hope that you find the referees' comments helpful when revising the manuscript for future submission elsewhere.

Yours sincerely,

Editor
The EMBO Journal

Referee reports:

Referee #1 (Remarks to the Author):

Comments for the authors:

Activation and recruitment of neutrophils to the site of infection is critical in host immunity to clearance of invading pathogens.
In this manuscript the authors report that a staphylococcal cysteine protease, Staphopain A, is a specific CXCR2 inhibitor. The enzyme affects neutrophil activation/chemotaxis by specifically cleaving the N-terminus of CXCR2, an important chemokine receptor that plays a role in recruitment of neutrophils to the site of infection. The authors have also identified the cleavage site in the N-terminal, extracellular region of CXCR2. This is a novel observation that expands our knowledge of strategies evolved in S. aureus to modulate and circumvent host defense systems.

Major concern:
The protease activity of Staphopain A seems to be fairly broad as it is reported to cleave a number of proteins, including fibrinogen, an abundant plasma protein. This broad specificity raises the question: Will CXCR2 cleavage occur also in vivo or in the presence of blood plasma where many other competing substrates are present? The observation reported in this study would be more significant if the authors could address this question.

Minor Comments:
1.Fig4A. To support the proposed mechanism of CXCR2 inactivation by Staphopain A the authors should use an antibody against the C-terminal of the CXCR2 to confirm the presence of the truncated CXCR2 on the cell surface after Staphophain A treatment.

2.Fig 4B and 5B. A higher quality of images should be provided. Two bands are present in the cleavage product of buffer condition shown in Fig4B. It is confusing which band the authors are referring to as the released CXCR2 fragment. The signal for the cleavage product shown in Fig5B is difficult to detect.

3.In the introduction section, GRO, ENA, NAP and GCP should be spelled out before abbreviation are used.

4.Page 4, 4th, 9th and 11th line from the bottom. References should be provided.

Referee #2 (Remarks to the Author):

Remarks to the author:

S. aureus is a successful human pathogen causing a large variety of diseases due to broad array of virulence factors. For the long time extracellular proteases of S. aureus were implicated to play the role in pathogenicity but this contention was somehow dwarfed by a general belief that their function is limited to generation of nutrients. Despite several recent publications showing potential mechanisms of action of staphylococcal proteases they are still neglected as important virulence factors in the mainstream publications on S. aureus. In this manuscript authors presented data that staphopain A may play an important immunomodulatory role is staphylococcal infection by disarming signaling via CXCR2. By specific cleavage of the N-terminal domain of CXCR2, Staphopain A makes neutrophils non-responsive to a whole set of this receptor-specific chemokines. This novel and interesting mode of interference with neutrophils accumulation and activation can be easily apprehended as deterrence against antibacterial mechanisms of innate immunity. As for many other immunomodulatory proteins of S. aureus, Staphopian A targets specifically human CXCR2.

This makes impossible the verification if the proteolytic inactivation of CXCR2 happens in vivo and has bearing on S. aureus pathogenicity in conventional models of murine infection.

The paper is very clearly written and conclusions drawn based on results of well designed and executed experiments are firm. Nevertheless, there are few points needing elucidation.

1) Staphopain A exerts its effect on CXCR2 at relatively high concentration (IC50 = ca. 40 nM for inhibiting CXCL1/CXCL7 induced calcium mobilization). There are no reports on concentration of staphylococcal proteases at the infection/colonization site so it is impossible to verify if staphopain A can reach this levels in vivo. In my opinion this should be commented in the paper. In addition, I suggest adding the reference to a publication by Jones et al. (J. Bacteriol. (2008) 190: 5265-5278) showing that the staphopains are the most abundantly produced proteases of S. aureus.
2) The lack of staphopain effect on binding of blocking mAbs does not necessary mean that recognized by them receptors are not cleaved. A cleavage of a receptor, which does not destroy a recognized epitope will not cause a drop in the level of mAbs binding. Please, comment on that.
3) Expression of the N-terminal region of CXCR2 in a context to a fusion partner protein may affect the structure of the polypeptide exposing a different cleavage site than that recognized in the native CXCR2. Would it be possible to immuoprecipitate (or purify in other way) the native N-terminally truncated receptor for analysis? Otherwise it may be helpful to refer to Staphopain A specificity which corroborates well with the identified LLD#A cleavage site (Dubin et al. Bochimie, doi: 10.1016/j.biochi.2011.07.020),
4) S. aureus developed many means to defend itself from attack by forces of innate immunity. How important is Staphopain A in comparison to other factors interfering with antibacterial activity of neutrophils? Does Staphopain A activity contributes to deterrence of neutrophils or is redundant, at least in the in vitro setting? In other words, I would like to see a comparison of effect of wild-type S. aureus and Staphopain A-null isogenic mutant growth media on CXCR2-dependent functions of neutrophils.

Minor point: Please add bibliographical details to reference 21

Referee #3 (Remarks to the Author):

The work of Laarman and colleagues investigates the contribution of StaphopainA, a Staphylococcus aureus cysteine protease in the proteolytic inactivation of the neutrophil (PMN)-specific chemokine receptors. The working hypothesis is that the protease StaphopainA inhibits the neutrophil recruitment and activation at the site of infection by cleaving the CXCR2 receptor. This study shows evidences that treatment of PMN with staphopainA decreases rapidly the capacity of neutrophils to bind a monoclonal antibody directed against the CXCR2 N-terminus and the intracellular calcium mobilization. Moreover, they found that this process is specific of human but not mouse PMN. Finally, they found that StaphopainA is able to cleave a recombinant fusion protein containing the CXCR2 N-terminus. The work is well performed but some major conclusions are not supported by experiments and so far are not enough convincing. Finally, the results are not yet supporting any significant role of this mechanism in a model of infection with S. aureus.

The major comment is that the authors claim that StaphopainA cleaves CXCR2 on freshly isolated PMN and the recombinant CXCR2-expressing cell line U937-CXCR2. Their experiments clearly show in vitro cleavage of the recombinant fusion protein CXCR2-GB1. There is no direct evidence that CXCR2 is cleaved in PMN since the authors use surface antibody staining and flow cytometry that did not assess internalization of CXCR2. Intracellular staining, confocal or fluorescence microscopy have to be used to address this point. The second aspect is that the specificity of cleavage in U937 cells should have been investigated with U937 cells expressing several unrelated receptors (human CXCR1 or mouse CXCR2). Such experiments will show whether StaphopainA cleave specifically human CXCR2. Finally, since the authors assume that the cleavage is specific for the N-terminal part of CXCR2, analysis with antibodies specific for C-terminus or polyclonal antibodies have to be used: such control will be instrumental for conclusions.

There are discrepancies and questions concerning the figure 1. The panel "a" displays fold inhibition of fluorescence (i.e. binding of fluorescent antibody). There is no indication on the real measure of fluorescence (gate in histogram, median, mean,). There is no statistics or comments on the number

of individual PMN samples that the study assessed. The panels "b" and "c" clearly show that the CXCR2 staining are variable: in "b", CXCR2-specific MFI of control "buffer" cells is about 50 and in "c" about 15. The ratio of MFI (fold inhibition) is respectively around 9 and 7. This also raises questions about the other receptors analyzed in the study. Depending on the magnitude of staining by antibodies, the calculation of inhibition may be biased. Low staining (i.e. close to background staining) may be difficult to associate to inhibition. Information on receptor staining (buffer) at homeostasis should be provided. Did the authors use isotype controls and FcR blocking reagents? In panel "a", is the receptor name fMLP or fMLP-R? The authors could have included StaphopainB as a positive control that is known to cleave CD11b and CD31: they comment briefly in results section about it but did not show any data.

In physiological conditions, one can assume that both chemokines and proteases will be present in the bacterial environment. In Figures, the authors treated the cells with StaphopainA +/- StaphostatinA (a specific inhibitor) and then assessed the effects on antibody surface staining, calcium mobilization, cell recruitment and signaling. Did the authors study the effect of pre-treatment or co-administration of chemokines on the activity of StaphopainA? Chemokine may over compete the effect of StaphopainA. Can CXCR2-specific antibody block the effect of StaphopainA? Finally, can the recombinant protein CXCR2-GB1-His compete with the effect of StaphopainA on PMN? Addressing this issue may support the idea that there is a physiological cleavage of CXCR2 on PMN. Similar experiments using supernatants of S. aureus (wild type or ScpA mutant) cultures combined with the supplementation of specific inhibitors of proteases (StaphopainA or StaphopainB...) will provide valuable data to conclude about the specific role of StaphopainA on CXCR2 cleavage.

The site of cleavage has been identified using the CXCR2-GB1-his recombinant protein. The homologous sequence in mouse is different and the authors propose that this is the reason why the effect of StaphopainA on S. aureus virulence cannot be assessed in mice. The authors should have mutated the recombinant protein at the site of cleavage with the sequence of mouse CXCR2. A recent study found that StaphopainA cleaves synthetic substrates with various sequences efficiently. The sequence identified in this study is significantly different. The authors should comment this recent observation. Can the purified StaphostatinA block the cleavage of CXCR2-GB1-His (as tested in Fig.4b)?

The second comment is about the physiology of S. aureus and the regulation of expression of its virulence factors. In the culture conditions used by the authors, did the bacteria secrete other proteases? Is the level of production similar and are the conditions optimal for secretion? In Fig.5b, the authors used undiluted supernatant of culture. Did they obtain similar results using concentrated supernatants as in Fig.5a or supernatants collected at different phase of cultures? There is a specific effect of StaphopainB on PMN apoptosis. Did StaphopainA induce similar effects on long term kinetic? How the different virulence factors impact the pathogenic effect: do the proteases specific for PMN synergize, antagonize or have additive effects on cleavage of receptors or other host proteins (matrix...), cell activation, signaling, recruitment, ...? These issues will really add value to the understanding StaphopainA effect on virulence.

The authors did not comment about the conservation of ScpA among the various isolates of S. aureus. Is the gene found in all isolates?

Minor comments:
1- The methods are divided in a too large number of sections.
2- The calculation and the measure of fluorescence are not clearly specified (MFI for flow cytometry, Fluo3calcium, ...)
3- The duration of Fluo3 assay is not specified
4- Statistical analyses is not presented (type of test, number of biological or technical replicates...)
5- Why do Histidine tag-specific antibodies in Fig.5b detect StaphostatinA?

Re-submission 26 April 2012

Hereby we send you our extensively revised manuscript entitled "*Staphylococcus aureus* Staphopain A inhibits CXCR2 dependent neutrophil activation and chemotaxis".
We were pleased that the referees showed interested in our study. We addressed all concerns raised by the referees, performed the suggested additional experiments and extensively revised and thereby improved our manuscript. The new manuscript contains new experimental data answering the following major concerns:
-Confocal and flow cytometry data demonstrating the specificity of Staphopain A for the N-terminus of CXCR2 (Figure 5B-D)
-Western analysis demonstrating that Staphopain A can cleave CXCR2 in the presence of human plasma (Fig 6D)
-Expression data of Staphopain A during *S. aureus* growth (Fig 7A)
-Data showing that Staphopain A is unique among *S. aureus* proteases to cleave CXCR2 (Fig 7A-B, Supplemental Figure 4A-C)
-Experiments addressing the specificity of Staphopain A for human versus mouse CXCR2 (Fig 6C, Supplemental Figure 3A-B)
Furthermore, we performed experiments and made textual changes to address other minor concerns raised by the referees.

In the letter below we have outlined how the paper is revised and furthermore we answer the concerns that were raised by the referees in a point-to-point reply. The exact changes to the original paper are highlighted in yellow in the revised version, which is submitted as the research article Laarman_REVISED. New figures are also highlighted in yellow.
We trust that you find our revised version of the manuscript suitable for publication in *EMBO Journal*.

POINT-TO-POINT REPLY

<u>Referee #1 raises 5 points:</u>

Point 1:
(major concern) The protease activity of Staphopain A seems to be fairly broad as it is reported to cleave a number of proteins, including fibrinogen, an abundant plasma protein. This broad specificity raises the question: Will CXCR2 cleavage occur also in vivo or in the presence of blood plasma where many other competing substrates are present? The observation reported in this study would be more significant if the authors could address this question.

Answer 1:
We tested whether CXCR2 cleavage also occurs in the presence of blood pasma. Therefore, $CXCR2_{1-48}$-GB1-His protein was incubated with Staphopain A in the presence of human plasma. Although plasma slightly competes with the cleavage, we found that Staphopain A can still cleave CXCR2 in 10% plasma. These data are included in the revised manuscript as figure 6D.

Point 2:
Fig4A. To support the proposed mechanism of CXCR2 inactivation by Staphopain A the authors should use an antibody against the C-terminal of the CXCR2 to confirm the presence of the truncated CXCR2 on the cell surface after Staphophain A treatment.

Answer 2:
We performed the following experiments to show that Staphopain A specifically cleaves the N-terminus of the receptor, leaving the rest intact:
1) We used Human Embryonic Kidney (HEK) cells expressing the human CXCR2 receptor modified with a C-terminal fusion to Yellow Fluorescent Protein (YFP). These cells were treated with Staphopain A and the N-terminus was stained with a monoclonal antibody.

Using confocal microscopy, we showed that Staphopain A specifically removed the N-terminus since the antibody could no longer detect the receptor, while the C-terminal YFP fluorescence was still intact. These experiments are presented in figure 5B of the revised manuscript
2) On neutrophils, we used an antibody directed against the third extracellular loop of CXCR2. We tried to use commercial antibodies against the C-terminus of CXCR2, as the referee suggested, but these didn't show specific binding in our hands. When Staphopain A was incubated with neutrophils, we observed that the antibody against the extracellular loop could still bind, showing that the rest of the receptor is still intact. These experiments are presented in figure 5C&D of the revised manuscript.

Point 3:
Fig 4B and 5B. A higher quality of images should be provided. Two bands are present in the cleavage product of buffer condition shown in Fig4B. It is confusing which band the authors are referring to as the released CXCR2 fragment. The signal for the cleavage product shown in Fig5B is difficult to detect.

Answer 3:
1) Fig 4B: we repeated this experiment using a more pure preparation of the CXCR2 peptide resulting in a clear image. This experiment is presented in the revised manuscript as figure 6A.
2) Fig 5B: The new figures 7B and 7C with supernatant cleavage of CXCR2 protein show very clear cleavage products.

Point 4:
In the introduction section, GRO, ENA, NAP and GCP should be spelled out before abbreviation are used.

Answer 4:
This is now corrected.

Point 5:
Page 4, 4th, 9th and 11th line from the bottom. References should be provided.

Answer 5:
These references are now included.

<u>**Referee #2 raises 5 points:**</u>

Point 1:
Staphopain A exerts its effect on CXCR2 at relatively high concentration (IC50 = ca. 40 nM for inhibiting CXCL1/CXCL7 induced calcium mobilization). There are no reports on concentration of staphylococcal proteases at the infection/colonization site so it is impossible to verify if staphopain A can reach this levels in vivo. In my opinion this should be commented in the paper. In addition, I suggest adding the reference to a publication by Jones et al. (J. Bacteriol. (2008) 190: 5265-5278) showing that the staphopains are the most abundantly produced proteases of S. aureus.

Answer 1:
We agree with the referee that it is impossible to determine the levels of Staphopain A during an infection *in vivo*. The only estimate that can be given are the enzyme levels in supernatants of liquid cultures *in vitro*. To analyse the native levels of Staphopain A in bacterial supernatant we titrated the Staphostatin A inhibitor into the supernatant of wild-type USA300 and analysed CXCR2 cleavage. Since Staphostatin A is highly specific for Staphopain A and forms a 1:1 complex, it can be used for this purpose. Staphopain A activity was partially inhibited at 50 nM MBP-ScpB and completely inhibited at 200 nM, suggesting that *S. aureus* secretes levels of ScpA that range from 50-200 nM. These concentrations are 1.25-5x higher than the determined IC_{50} for CXCR2 cleavage (40 nM). These data are included in the revised manuscript as figure 7C.
We discuss these results in the Discussion section and also included the publication by Jones et

al, as suggested by the referee.

Point 2:
The lack of staphopain effect on binding of blocking mAbs does not necessary mean that recognized by them receptors are not cleaved. A cleavage of a receptor, which does not destroy a recognized epitope will not cause a drop in the level of mAbs binding. Please, comment on that.

Answer 2:
The following section was included in the Discussion of the revised manuscript: "Our multi-screening antibody assay indicated that the interaction of Staphopain A with neutrophils is highly specific for CXCR2. All antibodies were selected for recognizing important epitopes, however a negative result in this antibody assay does not exclude that Staphopain A might also cleave other neutrophil receptors."

Point 3:
Expression of the N-terminal region of CXCR2 in a context to a fusion partner protein may affect the structure of the polypeptide exposing a different cleavage site than that recognized in the native CXCR2. Would it be possible to immuoprecipitate (or purify in other way) the native N-terminally truncated receptor for analysis? Otherwise it may be helpful to refer to Staphopain A specificity which corroborates well with the identified LLD#A cleavage site (Dubin et al. Bochimie, doi: 10.1016/j.biochi.2011.07.020),

Answer 3:
We agree with the referee that the structure of the CXCR2-GB1-His protein may be different from the native N-terminus expressed on cells. We included a comment on this in the Discussion of the revised manuscript.
Unfortunately, experiments to purify the native N-terminally truncated receptor failed due to technical reasons. According to the referees' suggestion, we included the following section in the Discussion commenting on the Kalinska&Dubin paper:
"We identified LLD↓A as the Staphopain A cleavage site in CXCR2, a finding that largely corresponds with a recent study using CLiPS that showed that Staphopain A prefers a Leucine at the P2 substrate subsite. Further, this study indicated that Staphopain A has no preferential P3 site and that Staphopain A, like other Staphopain enzymes, prefers a residue with small side chains in the P1 (Gly) and P1' (Ala or Ser) position. The LLD↓A site also contains an Alanine at the P1' site. Although CliPS didn't reveal an Aspartic acid at P1, it should be noted this amino acid has a relatively small side chain."

Point 4:
S. aureus developed many means to defend itself from attack by forces of innate immunity. How important is Staphopain A in comparison to other factors interfering with antibacterial activity of neutrophils? Does Staphopain A activity contributes to deterrence of neutrophils or is redundant, at least in the in vitro setting? In other words, I would like to see a comparison of effect of wild-type S. aureus and Staphopain A-null isogenic mutant growth media on CXCR2-dependent functions of neutrophils.

Answer 4:
Unfortunately, a comparison of bacterial supernatants in cellular assays is impossible due to the cellular toxicity of other secreted *S. aureus* (toxins, haemolysins, PSMs, unknown factors). Therefore, we compared supernatants of wild-type and mutant *S. aureus* in cleavage assays with purified CXCR2 N-terminus convincingly showing that Staphopain A is unique among *S. aureus* proteases to cleave CXCR2.These results are presented in figures 7A-7B.

Furthermore, to address the concern about the importance of Staphopain A in relation to other *S. aureus* neutrophil inhibitors, we included the following section in the Discussion of the revised manuscript:
"The function of Staphopain A, blocking neutrophil activation and chemotaxis, seems redundant next to the other Staphylococcal chemotaxis inhibitors. However, Staphopain A exclusively inhibits neutrophil activation via CXCR2 stimuli. Since a large number of different chemoattractants mediate neutrophil influx to the infected tissue, *S. aureus* benefits from an

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elaborate army of chemotaxis inhibitors that target different receptors. Likely, these inhibitors all act in concert to effectively down-modulate the inflammatory response and enhance the bacterial chances for survival. We predict that taking away only a few of these inhibitors would greatly reduce that bacterial virulence. This hypothesis is strongly supported by a recent study in which we mutated two *S. aureus* complement inhibitors and analysed bacterial virulence in various mouse models. Even though *S. aureus* expresses a large number of complement inhibitory proteins, taking away two of these factors greatly reduced bacterial pathogenesis."

Point 5:
Please add bibliographical details to reference 21

Answer 5:
This is now corrected.

<u>**Referee #3 raises 17 points:**</u>

Point 1:
The major comment is that the authors claim that StaphopainA cleaves CXCR2 on freshly isolated PMN and the recombinant CXCR2-expressing cell line U937-CXCR2. Their experiments clearly show in vitro cleavage of the recombinant fusion protein CXCR2-GB1. There is no direct evidence that CXCR2 is cleaved in PMN since the authors use surface antibody staining and flow cytometry that did not assess internalization of CXCR2. Intracellular staining, confocal or fluorescence microscopy have to be used to address this point. The second aspect is that the specificity of cleavage in U937 cells should have been investigated with U937 cells expressing several unrelated receptors (human CXCR1 or mouse CXCR2). Such experiments will show whether StaphopainA cleave specifically human CXCR2. Finally, since the authors assume that the cleavage is specific for the N-terminal part of CXCR2, analysis with antibodies specific for C-terminus or polyclonal antibodies have to be used: such control will be instrumental for conclusions.

Answer 1:
<u>**Specificity for the N-terminus (also see Point 2 of Referee 1):**</u>
1) Using HEK cells expressing CXCR2 with a C-terminal YFP tag, we determined by confocal microscopy that the C-terminal part of CXCR2 is still intact. In these cells we didn't observe that Staphopain A induces internalization of the receptor. These experiments are presented in figure 5B of the revised manuscript
2) Also on neutrophils we could demonstrate that Staphopain A specifically cleaves the N-terminus of CXCR2. An antibody directed against the third extracellular loop of CXCR2 could still bind to its receptor after treatment with Staphopain A. This also demonstrates that the rest of the receptor is still on the membrane and not internalized. These experiments are presented in figure 5C (flow cytometry) and 5D (confocal microscopy) of the revised manuscript.
<u>**Specificity for human CXCR2 versus human CXCR1:**</u>
1) In HEK cells, we also used constructs expressing human CXCR1. We observed that Staphopain A did not cleave this receptor (figure 5B of the revised manuscript)
2) On neutrophils, we also used antibodies recognizing the N-terminus of human CXCR1. We observed that Staphopain A did not cleave this receptor on neutrophils (figure 5C and 5D of the revised manuscript)
<u>**Specificity for human CXCR2 versus mouse CXCR2:**</u>
1) Murine neutrophils were treated with Staphopain A and stained with an antibody directed against the N-terminus of murine CXCR2. In contrast to human neutrophils, Staphopain A did not reduce the binding of this antibody (Supplemental figure 3B of the revised manuscript).
2) We repeated the activation assay on murine neutrophils now using varying concentrations of murine KC. Staphopain A did not inhibit activation of murine CXCR2 (Supplemental figure 3A of the revised manuscript)

Point 2:

There are discrepancies and questions concerning the figure 1. The panel "a" displays fold inhibition of fluorescence (i.e. binding of fluorescent antibody). There is no indication on the real measure of fluorescence (gate in histogram, median, mean,). There is no statistics or comments on the number of individual PMN samples that the study assessed. The panels "b" and "c" clearly show that the CXCR2 staining are variable: in "b", CXCR2-specific MFI of control "buffer" cells is about 50 and in "c" about 15. The ratio of MFI (fold inhibition) is respectively around 9 and 7. This also raises questions about the other receptors analyzed in the study. Depending on the magnitude of staining by antibodies, the calculation of inhibition may be biased. Low staining (i.e. close to background staining) may be difficult to associate to inhibition. Information on receptor staining (buffer) at homeostasis should be provided. Did the authors use isotype controls and FcR blocking reagents?
In panel "a", is the receptor name fMLP or fMLP-R? The authors could have included StaphopainB as a positive control that is known to cleave CD11b and CD31: they comment briefly in results section about it but did not show any data.

Answer 2:
Variability in receptor expression: We agree that there is variation in the receptor expression on cells of different donors on different days. To give a clear idea on the actual expression of other receptors we included a figure showing the 'Mean Fluorescence' data instead of 'fold inhibition'. This figure is presented as Supplemental Figure 1a.
Furthermore, we replaced the old Figure 1a in which we showed the data of one experiment with the new Figure 1a showing the mean+se of three independent experiments using different donors.
***To indicate the real measure of fluorescence, we included the following information to the Methods section: "The mean fluorescence of 10,000 gated neutrophils was determined and the relative expression was calculated by dividing the mean fluorescence of Staphopain A treated cells by buffer treated cells."**
***In the legends we now comment on the number of PMN samples assessed: "The experiment was repeated three times using different donors.".**
***Isotype controls didn't show binding to neutrophils indicating that the assay was not hampered by antibody binding to Fc receptors. In the Methods we included information stating that we used isotype controls.**
***We changed the name fMLP into FPR (Formylated Peptide Receptor)**
***We also performed this multiscreening antibody assay for Staphopain B. For unclear reasons, we didn't observe inhibited binding of our monoclonal antibodies against CD11b and CD31.**

Point 3:
In physiological conditions, one can assume that both chemokines and proteases will be present in the bacterial environment. In Figures, the authors treated the cells with StaphopainA +/- StaphostatinA (a specific inhibitor) and then assessed the effects on antibody surface staining, calcium mobilization, cell recruitment and signaling.
Did the authors study the effect of pre-treatment or co-administration of chemokines on the activity of StaphopainA? Chemokine may over compete the effect of StaphopainA. Can CXCR2-specific antibody block the effect of StaphopainA?
Finally, can the recombinant protein CXCR2-GB1-His compete with the effect of StaphopainA on PMN? Addressing this issue may support the idea that there is a physiological cleavage of CXCR2 on PMN.

Answer 3:
We studied the effect of co-administration of CXCL1 together with Staphopain A on CXCR2 cleavage and observed that Staphopain A can still cleave the CXCR2 receptor on cells in the presence of 10 nM CXCL1 (antibody binding assay). However, in cellular activation assays, the chemokine already triggers receptor activation before cleavage occurs. This indicates that Staphopain A needs to cleave its receptor prior to stimulus exposure. We expect that other staphylococcal chemotaxis inhibitors provide the time required for receptor cleavage by Staphopain A. *S. aureus* produces non-proteolytic, competitive inhibitors that block neutrophil recruitment via complement C5a (SCIN, Efb, Ecb, SSL7) or the bacterial product fMLP (CHIPS, FLIPr/FLIPr-like). C5a and fMLP are important for cellular recruitment in the first stage of an infection while chemokines coordinate the secondary, more specific influx.

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Therefore, Staphopain A can act adequately in the secondary phase, cleaving the receptor before the agonist is produced.
Since the monoclonal antibodies against CXCR2 are blocking antibodies, we could not analyse competition between these antibodies and Staphopain A. The antibody itself already down-modulates stimulation.
Recombinant CXCR2 peptide did not compete with the effect on neutrophils. This is not surprising since proteolytic enzymes are not "consumed" by their substrate and can only be blocked using irreversible inhibitors.

Point 4:
Similar experiments using supernatants of S. aureus (wild type or ScpA mutant) cultures combined with the supplementation of specific inhibitors of proteases (StaphopainA or StaphopainB...) will provide valuable data to
conclude about the specific role of StaphopainA on CXCR2 cleavage.

Answer 4:
Please see Answer 4 of Referee 2.

Point 5:
The site of cleavage has been identified using the CXCR2-GB1-his recombinant protein. The homologous sequence in mouse is different and the authors propose that this is the reason why the effect of StaphopainA on S. aureus virulence cannot be assessed in mice. The authors should have mutated the recombinant protein at the site of cleavage with the sequence of mouse CXCR2.

Answer 5:
As suggested by the referee, we mutated the CXCR2 protein changing the Leucine at position 34 into a Proline. Indeed, we observed less potent cleavage of this mutated protein by Staphopain A. However, there was still cleavage suggesting that other parts of the receptor than the cleavage site determine Staphopain A specificity. This experiment is now included in the revised manuscript as figure 6c and we added a comment discussing the differences in the Discussion section.

Point 6:
A recent study found that StaphopainA cleaves synthetic substrates with various sequences efficiently. The sequence identified in this study is significantly different. The authors should comment this recent observation.

Answer 6:
Also see Answer 3 of Referee 2.
We included a section in the discussion commenting on the paper by Kalinska&Dubin studying the substrate specificity of Staphopain A using different synthetic substrates and a bacterial protein substrate library (CLiPS). As the authors indicated in this paper, the synthetic substrate libraries didn't yield consistent data on Staphopain specificity. Using CliPS, the following substrate sites were found to be important for Staphopain A specificity: 1) the P2 site must be Leucine; 2) Staphopain A has no preferential P3 site; 3) like the other Staphopain enzymes, Staphopain A prefers a residue with small side chains in the P1 and P1' position. As already indicated by Referee 2, these findings corrobate well with the LLD#A cleavage site in CXCR2.

Point 7:
Can the purified StaphostatinA block the cleavage of CXCR2-GB1-His (as tested in Fig.4b)?

Answer 7:
Yes, Staphostatin A blocks the cleavage of CXCR2-GB1-His. We repeated this experiment (see referee 1, point 3) and included this control in the new experiment. (Figure 6A of the revised manuscript).

Point 8:
The second comment is about the physiology of S. aureus and the regulation of expression of its

virulence factors. In the culture conditions used by the authors, did the bacteria secrete other proteases? Is the level of production similar and are the conditions optimal for secretion?

Answer 8:
As suggested by the referee, we analysed the growth-phase dependent production of Staphopain A. Wild-type and ScpA mutant bacteria were grown in liquid cultures and supernatants were collected at different time points of the growth curve, incubated with the CXCR2 fusion protein, and proteolytic cleavage was analysed by immunoblotting. At certain points in the growth phase we observed complete cleavage of the CXCR2 protein without concentrating the supernatants. Using a Staphostatin titration, we could show that the levels of Staphopain range from 50-200nM. Using various known substrates for *S. aureus* enzymes, we showed that other proteases are secreted as well. For SplB we showed that the concentrations are lower than Staphopain A. These experiments are presented in Figure 7A-C and in Supplementary figure 4.

Point 9:
In Fig.5b, the authors used undiluted supernatant of culture. Did they obtain similar results using concentrated supernatants as in Fig.5a or supernatants collected at different phase of cultures?

Answer 9:
In contrast to the experiments in Fig. 5a of the initial manuscript, we now observed that cleavage of CXCR2 can go to completion with undiluted supernatants. We incubated 3h-supernatants with the CXCR2 protein for several time points. Within 1 hour, all CXCR2 protein was converted to product. Supernatants of ScpA mutant bacteria did not convert the substrate and complementation of the mutant resorted activity to wild-type levels. These data are presented in figure 7b.

Point 10:
There is a specific effect of StaphopainB on PMN apoptosis. Did StaphopainA induce similar effects on long term kinetic?

Answer 10:
We analysed whether Staphopain A can induce similar effects on PMN apoptosis. Therefore, neutrophils were incubated with Staphopain A or Staphopain B for 75 minutes at 37°C and binding of Annexin V or Propidium Iodide was monitored. In contrast to Staphopain B, Staphopain A did not induce binding of Annexin V or Propidium Iodide. These data are presented in supplemental figure 1b.

Point 11:
How the different virulence factors impact the pathogenic effect: do the proteases specific for PMN synergize, antagonize or have additive effects on cleavage of receptors or other host proteins (matrix...), cell activation, signaling, recruitment, ...? These issues will really add value to the understanding StaphopainA effect on virulence.

Answer 11:
Among *S. aureus* proteases, only the Staphopains have been shown to act on neutrophils. It seems tempting to speculate that the Staphopains might work together in modulating neutrophil functions. We feel these investigations go beyond the scope of this paper and will be subject of further studies. However, we included a comment on this issue in the Discussion section.

Point 12:
The authors did not comment about the conservation of ScpA among the various isolates of S. aureus. Is the gene found in all isolates?

Answer 12:
In the Introduction, we now included a comment describing a study showing that ScpA is highly conserved among *S. aureus* isolates.

Point 13:

The methods are divided in a too large number of sections.

Answer 13:
We merged some of the methods sections.

Point 14:
The calculation and the measure of fluorescence are not clearly specified (MFI for flow cytometry, Fluo3calcium, ...)

Answer 14:
In the methods section ("flow cytometry) we have included more detailed information on how the different measures for fluorescence were calculated.

Point 15:
The duration of Fluo3 assay is not specified

Answer 15:
In the Methods section we now provide more background information on how the Fluo3 calcium mobilization assay was exactly performed, also specifying the duration of the assay.

Point 16:
Statistical analyses is not presented (type of test, number of biological or technical replicates...)

Answer 16:
The applied statistical analyses is now presented in the methods section. Further, we ensured that all legends contain information about the number of replicates and type of test.

Point 17:
Why do Histidine tag-specific antibodies in Fig.5b detect StaphostatinA?

Answer 17:
In the old Fig. 5b we used commercially obtained Staphostatin A, that seemed to cross-react aspecifically with the anti-His antibody. We repeated the Immunoblot now using different concentrations of MBP-tagged Staphostatin A that we made ourselves (Figure 7C). The MBP-labeled Staphostatin A did not show aspecific binding to the anti-His antibody.

2nd Editorial Decision 31 May 2012

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees express interest in your manuscript and at least two are broadly in favor of publication, pending satisfactory minor revisions.

At this stage I would like to draw your attention to the scarce statistical analysis provided that needs to be strengthen, as highlighted by referees #2 and #3.

I also would appreciate if you could discuss in the main text the limitations of experimenting with S. aureus to reflect the limited physiological/in vivo evidence offered by the study, thereby clarifying Referee #2's point.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

Referee reports:

Referee #1 (Remarks to the Author):

The work is nicely done, and significantly strengthened, compared to a previous version to which I had been exposed.
The central issue for this reviewer remains originality. Among the large amount of molecular systems that have been identified in S. aureus to manipulate elements of the innate immune system, the current one, in spite of the quality of the experimental work and convincing character of the results, does not stand out for the readership of a journal like EMBO J. I sincerely think that such work is perfectly fitted at this stage for specialty journals of microbial pathogenesis.

Referee #2 (Remarks to the Author):

The study of Laarman and colleagues aims to define the contribution of the Staphylococcus aureus cysteine protease in virulence. The authors found that Staphopain A cleaves the neutrophil PMN-specific chemokine receptor CXCR2. These findings provide novelty, are of broad biological significance and have importance to the specific field. The evidences for the conclusions that are drawn are significant.

The hypothesis is that the protease Staphopain A inhibits the neutrophil recruitment and activation at the site of infection by cleaving the CXCR2 receptor. This study shows that PMN treatment with Staphopain A inhibits neutrophils to bind a monoclonal antibody directed against the CXCR2 N-terminus and the intracellular calcium mobilization by chemokines. Staphopain A cleaves efficiently a fusion protein containing the human CXCR2 N-terminus and GB1 but inefficiently a fusion protein containing the mouse CXCR2 N-terminus and GB1, thereby correlating with specificity of the protease. The authors give evidence that CXCR2 is cleaved in PMN since the authors use surface antibody staining with various antibodies and confocal microscopy with cells transfected with recombinant hCXCR2-YFP fusions. The study also investigated the activity of supernatants of S. aureus (wild type or ScpA mutant) on cleavage of hCXCR2-GB1 fusion protein.

The results are not yet supporting a role of this mechanism in a model of infection with S. aureus but the findings are of interest for the scientific community.

Minor comments:
The study does not show any information about the statistics of Figure 5. How many cells were counted?
The figure 6 (panel C) shows that mCXCR2-GB1 is cleaved. The ratio of cleavage is lower than

CXCR2-GB1 but significant. This result is well discussed but should be better commented in the results section.
The activity of Staphopain A on CXCR2 fusion protein is well studied: it gives rises to N-terminal proteolysis. Did the authors perform any experiments on native CXCR2 other than inhibition of antibody binding? Is it really possible to exclude that Staphopain A bind to N-terminus of CXCR2 preventing antibody binding rather than cleavage of the epitope?

Referee #3 (Remarks to the Author):

The manuscript by Laarman et al demonstrates a specific pathophysiological role for Staphopain A from S. aureus. The authors show that Staphopain A specifically cleaves CXCR2 in its N-terminal region, and that function effects neutrophil function. Overall the paper is well written and logical, and would be of interest to the readers of EMBO J. Some minor points still need to be addressed, however:

1. There seems to be a sparing use of statistics in the paper. For example, figure 1 contains none. It stated that the histogram is a representative example, but how reproducible is this? Some sense of SD or SEM would be helpful. 2f contains no statistical analysis, when it would seem appropriate. Figure 3 contains none, when it is a similar analysis to figure 2, which largely does.

2. The paper as written refers to things in the definitive, when sometime what is observed is a fold decrease or increase, rather than an absolute outcome. Indeed, there is no mention of fold changes in the paper at all. An example of this is in figure 4, where it is categorically stated that Staphopain A treatment abrogates phosphorylated ERK. This may be true for p ERK 1, but there is clearly p ERK 2 present in the treated lanes. Firstly, the authors should correct the text to reflect this, and then perhaps perform densitometry analysis to understand the level of reduction seen. Finally, some discussion of why it is not a complete abrogation should be included.

3. In figure 5b and 5d no control is included with the staphostatin, yet it is included in 5a and 5c.

4. For the cleavage experiments, why do the authors see the cleavage product in 6a without the addition of Staphopain A? Is the protein inherently unstable? Are the affects observed just as a result of incubation times?

5. Figure 5d seems unnecessary - the observed alterations in the presence of human serum, while nice, are not really so relevant to the topic at hand. Additionally, this analysis uses Staphopain concentrations (250 and 500) vastly in excess of that seen for other experiments, meaning that there could be questionable physiological significance to this.

6. To support the cleavage study it would be helpful to include a predicted topology plot for CXCR2 in the supplemental figures. Much time is spent discussing the relative domains, but it is hard to understand what this means without a figure to look at. A simple plot via SOSUI, or similar website would allow us to see predicted topology in the context of the membrane, and particularly reveal the specific site of cleave in the context of protein folding.

7. Figure 7 presents important data as it shows the finding is not just an in vitro artifact. I have some concerns about the way the bacteria were grown, as cells reaching post-exponential phase by 3h is strange, and not really consistent with the typical way one thinks about a S. aureus growth curve. This is perhaps a result of the way in which the cells were cultured. Taking overnight cells and inoculating new media at an OD of 0.2 should result in a lag phase of 1-2h. It would also result in the cells being asynchronous because of the relatively high inoculum of stationary phase cells into new media. This might perhaps explain why the authors see a decline in Staphopain in Staphopain activity between 3-24h. Indeed, this finding is not consistent with a variety of studies on these enzymes. Also, the suggestion that this is a result of Staphopain A autodegradation is inaccurate as a variety of proteomics studies present in the literature clearly show full length and abundant Staphopain A from S. aureus cultures older than 3h. The authors should revise the way this section is articulated, and tone down their claims.

8. Additionally for these studies, no information is given on how the supernatants were prepared.

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How much was collected, were they filter sterilized as is common for such approaches (if not, contaminating enzymes from other cellular fractions may influence results), were they concentrated, if so how much was performed to achieve these results, were reducing agents added to augment the activity of the cysteine protease?

1st Revision - authors' response 09 July 2012

Thank you very much for the review of our manuscript entitled "*Staphylococcus aureus* Staphopain A inhibits CXCR2 dependent neutrophil activation and chemotaxis". We are pleased that the referees were enthusiastic about our study. We have now revised our manuscript according to their and your concerns. We performed additional experiments and addressed all issues raised. Also, we extended the statistical analyses and discuss the limitations of our study regarding *in vivo* experiments.

In the letter below we answer the concerns that were raised by the referees in a point-to-point reply. The exact changes to the original paper are highlighted in yellow in the revised version, which is submitted online as the research article Rooijakkers_REVISED. We thank you for your constructive comments and hope you find our revised manuscript suitable for publication in *The EMBO Journal*.

POINT-TO-POINT REPLY

The editor raises two points:

Point 1:
At this stage I would like to draw your attention to the scarce statistical analysis provided that needs to be strengthen, as highlighted by referees #2 and #3.

Answer 1:
We have included the required statistical analyses (please see reply to both referees)

Point 2:
I also would appreciate if you could discuss in the main text the limitations of experimenting with S. aureus to reflect the limited physiological/in vivo evidence offered by the study, thereby clarifying Referee #2's point.

Answer 2: We now discuss these limitations in the Discussion section of the revised manuscript. Also, we included a sentence in the abstract stating the limitations for in vivo experiments.

Referee #2 raises 3 points:

Point 1:
The study does not show any information about the statistics of Figure 5. How many cells were counted?

Answer 1: In the revised manuscript, we included information regarding the statistics of Figure 5. For figure 5a we scanned three western blot images to allow densitometry quantification (result is described in the text). Figure 5c, showing a FACS quantification of the images generated in figure 5d, already contained statistical analysis data including mean values and standard errors. In the methods section we now describe how many cells were analysed for confocal microscopy (5b and 5d).

Point 2:
The figure 6 (panel C) shows that mCXCR2-GB1 is cleaved. The ratio of cleavage is lower than CXCR2-GB1 but significant. This result is well discussed but should be better commented in the results section.

Answer 2:
This is now corrected. We extended the results section describing figure 6C.

Point 3:
The activity of Staphopain A on CXCR2 fusion protein is well studied: it gives rises to N-terminal proteolysis. Did the authors perform any experiments on native CXCR2 other than inhibition of antibody binding? Is it really possible to exclude that Staphopain A bind to N-terminus of CXCR2 preventing antibody binding rather than cleavage of the epitope?

Answer 3: In the revised manuscript we present two new experiments showing that proteolytic activity of Staphopain A is essential for inhibition of CXCR2. We blocked the proteolytic activity of Staphopain A with E-64, a small-molecule cysteine protease inhibitor that specifically targets the active site cysteine. We observed that E-64 can reverse the effect of Staphopain A, both in antibody competition and functional inhibition of native CXCR2 on neutrophils. These experiments are presented in figure 1c and Supplemental figure 2a.
Further, the manuscript already included assays demonstrating that Staphopain A does not only block antibody binding to native CXCR2 on cells. Staphopain A also functionally inhibits the receptor on cells (calcium mobilization and chemotaxis (figures 2-4)).

<u>**Referee #3 raises 8 points:**</u>

Point 1:
There seems to be a sparing use of statistics in the paper. For example, figure 1 contains none. It stated that the histogram is a representative example, but how reproducible is this? Some sense of SD or SEM would be helpful. 2f contains no statistical analysis, when it would seem appropriate. Figure 3 contains none, when it is a similar analysis to figure 2, which largely does.

Answer 1:
As suggested by the referee, the revised manuscript now also includes statistical analyses for figures 1, figure 2f, figure 3, figure 4a, figure 5. Instead of using representative histograms for figure 1b and 1c, we now included graphs showing the mean values of three independent experiments including the standard error.

Point 2:
The paper as written refers to things in the definitive, when sometime what is observed is a fold decrease or increase, rather than an absolute outcome. Indeed, there is no mention of fold changes in the paper at all. An example of this is in figure 4, where it is categorically stated that Staphopain A treatment abrogates phosphorylated ERK. This may be true for p ERK 1, but there is clearly p ERK 2 present in the treated lanes. Firstly, the authors should correct the text to reflect this, and then perhaps perform densitometry analysis to understand the level of reduction seen. Finally, some discussion of why it is not a complete abrogation should be included.

Answer 2:
Throughout the paper we have expressed our data as fold decrease/increase. For the ERK blot, we have performed densitometry analyses to describe the level of reduction. This result is included in the text of the Results section. Staphopain A treatment caused a statistically significant reduction of both pERK1 and pERK2. Indeed the reduction is stronger for pERK1 (50%) than for pERK2 (25%). It is not really clear to us why we observed differential inhibition of pERK1 and pERK2, possibly this is caused by higher background levels of pERK2 in unstimulated cells. We now discuss this in the text.

Point 3:
In figure 5b and 5d no control is included with the staphostatin, yet it is included in 5a and 5c.

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Answer 3:
This control was included, but not presented to keep these figures concise and clear. However, we now mention this in the text.

Point 4:
For the cleavage experiments, why do the authors see the cleavage product in 6a without the addition of Staphopain A? Is the protein inherently unstable? Are the affects observed just as a result of incubation times?

Answer 4:
The observed cleavage is not a result of just incubation time, since the gel clearly shows that you require Staphopain A to degrade the fusion protein (upper band). The lower band present in the lane without Staphopain A is a contaminant from the purification that runs at the same height as the cleavage product. In contrast to the cleavage product, this band does not react with the anti-His and anti-CXCR2 antibodies, indicating that it is a different protein.

Point 5:
Figure 5d seems unnecessary - the observed alterations in the presence of human serum, while nice, are not really so relevant to the topic at hand. Additionally, this analysis uses Staphopain concentrations (250 and 500) vastly in excess of that seen for other experiments, meaning that there could be questionable physiological significance to this.

Answer 5:
We would prefer to keep this figure in the manuscript since it shows that Staphopain also cleaves CXCR2 in presence of competing substrates like fibrinogen, which is present in plasma.

Point 6:
To support the cleavage study it would be helpful to include a predicted topology plot for CXCR2 in the supplemental figures. Much time is spent discussing the relative domains, but it is hard to understand what this means without a figure to look at. A simple plot via SOSUI, or similar website would allow us to see predicted topology in the context of the membrane, and particularly reveal the specific site of cleave in the context of protein folding.

Answer 6:
The revised manuscript contains a topology plot for CXCR2 in the context of the membrane. This plot is presented in Supplemental figure 4.

Point 7:
Figure 7 presents important data as it shows the finding is not just an in vitro artifact. I have some concerns about the way the bacteria were grown, as cells reaching post-exponential phase by 3h is strange, and not really consistent with the typical way one thinks about a S. aureus growth curve. This is perhaps a result of the way in which the cells were cultured. Taking overnight cells and inoculating new media at an OD of 0.2 should result in a lag phase of 1-2h. It would also result in the cells being asynchronous because of the relatively high inoculum of stationary phase cells into new media. This might perhaps explain why the authors see a decline in Staphopain in Staphopain activity between 3-24h. Indeed, this finding is not consistent with a variety of studies on these enzymes. Also, the suggestion that this is a result of Staphopain A autodegradation is inaccurate as a variety of proteomics studies present in the literature clearly show full length and abundant Staphopain A from S. aureus cultures older than 3h. The authors should revise the way this section is articulated, and tone down their claims.

Answer 7:
We have toned down our claims with respect to the loss of enzyme activity in overnight cultures being due to Staphopain A autodegradation. Furthermore we discuss the observed discrepancy with proteomic studies.
We have also started cultures at a lower optical density (0.05) and have still seen maximal proteolytic activity in late log phase cultures. A recent study by Nickerson et al suggested that autodegradation of Staphopain A can occur. However, proteomic studies showed contrasting data. Possibly, the discrepancy can be explained by the fact that we were able to, for the first

time, analyze Staphopain activity thanks to the CXCR2 substrate. Further studies will be needed to resolve this issue.

Point 8:
Additionally for these studies, no information is given on how the supernatants were prepared. How much was collected, were they filter sterilized as is common for such approaches (if not, contaminating enzymes from other cellular fractions may influence results), were they concentrated, if so how much was performed to achieve these results, were reducing agents added to augment the activity of the cysteine protease?

Answer 8:
The Methods section of the revised manuscript now includes more detailed information regarding the collection of bacterial supernatants.

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