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S. aureus virulence attenuation and immune clearance mediated by a phage lysin-derived protein

Hang Yang, Jingjing Xu, Wuyou Li, Shujuan Wang, Junhua Li, Junping Yu, Yuhong Li, Hongping Wei

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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 28th Aug 2017

Thank you for submitting your manuscript (EMBOJ-2017-98045) to The EMBO Journal. I have now had a chance to read it carefully and to discuss it with my colleagues, and I am sorry to say that we cannot offer publication in The EMBO Journal.

Your analysis reports on an anti virulence role of the phage lysin derived PlyV12. In vitro and in vivo mouse work shows that the CBD of PlyV12 (V12CBD) binds to Staphylococci aureus and blocks its virulence. The findings also show that V12CBD can protect mice from methicillin resistant S. aureus (MRSA) infections. V12CBD is also shown to enhance macrophage activity and their ability to phagocytosis S. aureus. I appreciate that the findings add new insight. However, previous work has also provided support for that PlyV12 has lytic ability against Staphylococci strains. I see that the present work extends this finding and provides in vivo support for this, but that lysins can have anti virulence against MRSA has also been shown. While we appreciate the potential therapeutic implications of the findings, we also find that the conceptual advance provided is not sufficient to consider publication in The EMBO Journal.

Please note that at the EMBO Journal we subject to external review only those submissions that have a high chance of timely acceptance. I am sorry to disappoint you on this occasion and wish you success with the rapid publication of this dataset elsewhere.

Resubmission 4th Oct 2017

I would like to thank you for your careful reading and valuable suggestions on our previous manuscript entitled "S. aureus virulence attenuation and immune clearance mediated by a phage lysin-derived protein" that has been submitted to The EMBO Journal about one month ago (Manuscript ID EMBOJ-2017-98045).

In the previous manuscript, we found for the first time that a phage lysin derived cell-wall binding protein (V12CBD) could sensitize S. aureus to immune clearance, change its virulence factors expression profile, activate host innate immunity, and protect mice from lethal MRSA challenges in therapeutic and prophylactic models. However, the molecular mechanisms underlying its activation of host immunity were not studied. In this resubmitted manuscript, we added more data to show that V12CBD can activate macrophage through NF-κB pathway. These include the qRT-PCR detection of cytokines using inhibitors targeting NF-κB and MAPKs pathways, in-situ detection of phospho-NF-κB-p65 by flow cytometry, and characterization of macrophage surface molecules, such as CCR7, MR, MHCII, CD80 and CD86. Although the detailed information on molecules that recognize and transfer V12CBD signaling needs further study, the current data clearly revealed that V12CBD activated macrophages through NF-κB pathway and thus enhanced host innate immune defense against invaded pathogens.

I hope the present manuscript meets the high quality requirements of your journal, and looking forward to hearing from you soon!

Editorial correspondence 16th Oct 2017

Thank you for submitting your manuscript to The EMBO Journal. I have now had a chance to take a look at the introduced changes.

I do appreciate the link to NF-KB and see that this adds new insight although the mechanism for how NF-kB is activated is not further explored. I decided to ask for advice on the manuscript from a good expert in the field. The advisor finds the manuscript potentially interesting, but also indicates that more support for the reported mechanism is needed.

In particular, the advisor raises 2 points

1) Regarding the impact of V12CBD on host cells and NFkappaB activation=> V12CBD is expressed and purified from E.coli. However, activation of NFkappaB might come from contaminating LPS endotoxin. Although it is stated that the protein preps are passed down an endotoxin removing gel, there is no data provided to show that LPS removal was checked afterwords.

2) There is little information on how a lysin CBD that binds to peptidoglycan affects the expression of Staph. aureus genes. V12CBD is stated to downregulate virulence genes, but when looking at the looking at the table of differentially expressed genes they are almost all metabolic with very few classical Staph. aureus cell wall or secreted virulence factors. The RNAseq data is validated with qRT-PCR, but there are no phenotypic assays to confirm that the fold changes in gene expression result in down-regulation genes that contribute to pathogenesis.

I presume you have data on point # 1 as this is an important control. Do you have any data to support the second point? We would need some insight along those lines for consideration here. If you can add such data then I am willing to send out the manuscript for full review.

I hope that you find these comments useful.

2nd Editorial Decision 22nd Dec 2017

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by 3 referees and their comments are provided below.

As you can see the referees find the analysis interesting. However, it is also clear that further experiments are needed to support the key conclusions of the paper. Should you be able to address the concerns raised in a significantly revised version that I am interested in considering a revised manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the raised concerns at this stage.

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://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

In the age of antibiotic resistance, phage therapy has received substantial interest as an alternative therapeutic strategy. It is in this context reasonable to explore the possibility that fragments of phages can have antimicrobial activities. In the current study the authors have used a recombination version of the Cell Binding Domain (CBD) of phage that target S.aureus and propose that this fragment affect bacterial attachment to and invasion of cultured A459 cells. In addition the recombinant protein preparation reduced bacterial survival in Macrophages and enhanced mouse survival in a peritoneal challenge model.

The problem with this study is that it does not include appropriate controls. There are two major concerns: 1) A His-tag protein purified in one step on a Ni-charged column is not pure. The E. coli lysate contain many molecules (In addition to LPS) that are recognized by the innate immune system and can trigger the responses seen in the macrophage and in vivo studies and reported in this manuscript. It is possible that these type of molecules contaminate the V12CBD preparation. 2) We know nothing about the biophysical properties of the V12CBD protein. Could the effects on S. aureus attachment and invasion of A459 cells be non-specific? A proper control could be a similar segment of a phage (with similar charge and hydrophobic properties) that does not target S. aureus. Such a control protein should be expressed and purified the same way as the test substance.

Referee #2:

This study investigates the capacity of a cell-wall binding domain fragment from a phage lysin to alter the host response and subsequent virulence of Staphylococcus aureus. The recombinant protein was capable of altering uptake of S. aureus into both epithelial and professional phagocytic cells. The lysin protein also altered expression of S. aureus genes, increased proinflammatory cytokine production in response to S. aureus and reduced mortality in a murine model of infection (using two different strains of S. aureus). There are currently no vaccines or no non-antibiotic treatments approved for use against S. aureus so novel treatment options are highly desired. The development of a lysin/recombinant protein approach is novel. There are concerns on the mechanism behind the lysin that require attention that need to be addressed and thus strengthen the validity of the claims in the study.

Major concerns

A major concern revolves around the action of the lysin protein. It has been shown that the lysin is lytic to S. aureus. The authors need to demonstrate that the CBD domain of the protein used in this study is not lytic, so that the mechanism explored in the study are not due to S. aureus being killed. All the data could be easily attributed to lysed bacteria.

First section of results, untreated vs treated. Could the result be just due to residual protein left on epithelial cells?

In figure 1 it is shown that the CBD influences both adhesion and invasion. Could the data from the invasion aspect be just a direct correlation to the adhesion data. There are probably less intracellular bacteria as a consequence of less bacteria adhering and being taken up by the cell.

The inhibitory capacity of CBD was also examined with macrophages. Is CBD capable of permeating cell membrane or being taken up by cells. Showing that CBD does not permeate the cell will demonstrate it is interfering with the phagocytic process and is not interfering with a function inside the cell.

Signaling in response to S. aureus in the presence/absence of V12CBD was tested with polymyxin B to prevent signaling as a result of contaminating LPS. This could alternatively be done using LPS from Rhodbacter sphaeroides. The control experiment showing that polymyxin B is working (preventing signaling from LPS) is missing.

The point was made with signaling in response to S. aureus and V12CBD that it was NF-kB dependent. However, non NF-kB dependent genes were quantified.

Minor concerns

Page 4 in the introduction, it is noted there are no antibody-based therapies for S. aureus. Some reference to the work being done with alpha toxin which is in phase 2/3 clinical trials should be noted.

Fig. 5b and f the data should be ordered the same way in each panel

Several spelling and grammatical errors were noted. The paper would benefit from editing by a native English speaker.

Referee #3:

In this manuscript, Yang et al. demonstrate that treatment of Staphylococcus aureus with a recombinant protein derived from the cell wall binding domain of phage lysin PlyV12 (V12CBD) results in reduced virulence gene expression, reduced invasion of epithelial cells and increased susceptibility to killing by macrophages. Incubating macrophages with V12CBD leads to their activation and enhanced phagocytic activity. Furthermore, authors show that both therapeutic as well as prophylactic administration of V12CBD protects mice from systemic S. aureus infection. The subject of this paper is of high importance, and the authors report very interesting findings. Unfortunately, some sections of the manuscript are poorly written and would benefit from careful editing by a native speaker. Furthermore, authors often fail to explain their figures and ought to be more careful with their data interpretation (e.g Fig 5d-e). Thus, there are some major concerns the authors need to address.

Major concerns:
• Some further detail on the strains used would be suggested. Please reference them in Materials & Methods (N315 PMID:11418146 and T23).
• P4 Line 86 contains incorrect citations. These papers referenced here are not on S. aureus vaccines.

• Figure 1c: What is the blue versus red line? This is not mentioned in main text nor in figure legend. I would suggest labeling graphs as Fig 1c) example FACS plot and Fig 1d) summary data of phagocytosis
• Figure 1g: Which strain was used for infection? Main text says T23, while Figure legend says N315.
• Figure 3d: greatest effect is seen with 100ug V12CBD. Why do authors choose to look at 25 and 50ug treatment?
• Figure 3f: please provide example FACS blot. What is the positive control here? Why do authors choose to treat with 100ug V12CBD here but with 25-50ug in Fig 3d?
• Figure 3g: Signaling inhibitors depicted in graph are neither mentioned in figure legend nor explained in text.
• It would make more sense if Supplemental Fig 3 would proceed Supplemental Fig 4 in main text as well as in order of figures.
• Supplemental Fig 6: NFkB detection is not visible in this graph. Please change X-axis to log scale.
• Figure 5b and Page 15, Line 320-322: since authors only used 10ul (a rather small volume) of 1ml organ homogenates to determine bacterial loads some of their samples were negative for CFU. I would suggest the authors plate the whole organ after not detecting any bacteria in their assay.
• Figure 5d and Page 15, Line 327: Text says TNFa levels are reduced after V12CBD treatment however graph shows an increase in TNFa (open circles) after V12CBD treatment. Did authors look at any other pro-inflammatory cytokines besides TNFa?
• Figure 5e and Page 15-16, Line 329-331: Since there is clearly increased production of TNFa the conclusion that there is less inflammatory injury in organs because of reduced TNFa levels does not make any sense. Please elaborate on histology images. What are we looking at besides that these are kidney and liver sections?
• Fig 5g) Please show weight changes post infection as % weight loss over time
• Page 14-15, Line 308-309: Authors should not conclude from a cytotoxicity testing in vitro to safety in vivo, instead should test if a high concentration of V12CBD in vivo has an adverse effect in mice.

Revision - authors' response 18th Mar 2018

Response to Reviewers Comments
Referee #1:
In the age of antibiotic resistance, phage therapy has received substantial interest as an alternative therapeutic strategy. It is in this context reasonable to explore the possibility that fragments of phages can have antimicrobial activities. In the current study the authors have used a recombination version of the Cell Binding Domain (CBD) of phage that target S. aureus and propose that this fragment affect bacterial attachment to and invasion of cultured A459 cells. In addition the recombinant protein preparation reduced bacterial survival in Macrophages and enhanced mouse survival in a peritoneal challenge model.

The problem with this study is that it does not include appropriate controls. There are two major concerns: 1) A His-tag protein purified in one step on a Ni-charged column is not pure. The E. coli lysate contain many molecules (In addition to LPS) that are recognized by the innate immune system and can trigger the responses seen in the macrophage and in vivo studies and reported in this manuscript. It is possible that these type of molecules contaminate the V12CBD preparation.
Response: We did two additional control experiments to exclude the effects of contaminated molecules during V12CBD preparation. 1) We purified another his-tagged phage lysin derived protein, the catalytic domain of lysin Ply187 (Pc, p*I*=9.47), under the same purification conditions. The prophylactic efficacy of Pc was tested on *S. aureus* T23 infected mouse model, which did not show the preventive effect as that of V12CBD (see **Figure 1 below**). 2) We cloned V12CBD coding sequence into pcDNA3.1(+) vector and transfected pcDNA3.1-*V12CBD* into BHK-21 cells to allow the expression of V12CBD protein within the cells. This experiment would not generate any molecules possible in the *E. coli* lysate. Upregulations of *Il1b*, *Il6*, *Tnfa* and *iNos* were observed in BHK-21 cells at 48 h after the transfection of pcDNA3.1-*V12CBD* compared with cells transfected with pcDNA3.1 vector alone. We added these data to the revised manuscript as **Fig 3g**.
Although these two additional experiments further confirmed the stimulation effect of V12CBD protein on the expressions of *Il1b*, *Il6*, *Tnfa* and *iNos*, we think your concern is reasonable considering that positively charged V12CBD (p*I*=9.96) would non-specifically bind with some

negatively charged molecules in *E. coli* lysate during our preparation, a situation that could not be absolutely excluded. To be more objective, we added a statement about this concern in the discussion section of the revised manuscript (pages 19-20, lines 415-419).

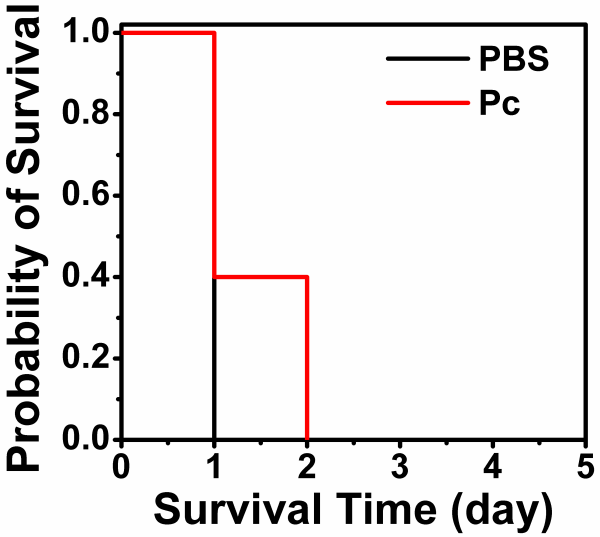

Figure 1 Survival of mice pre-treated with Pc. Mice are received intraperitoneal injection at a single dose of 12 mg/kg Pc (n=5), or equal volumes of PBS (n=5). Twenty-four hours later, mice are received a lethal dose of 2.2×10^8 CFU of *S. aureus* T23. The mortality of each group is recoded every day.

2) We know nothing about the biophysical properties of the V12CBD protein. Could the effects on S. aureus attachment and invasion of A459 cells be non-specific? A proper control could be a similar segment of a phage (with similar charge and hydrophobic properties) that does not target S. aureus. Such a control protein should be expressed and purified the same way as the test substance.
Response: V12CBD is the cell-wall binding domain of the phage lysin PlyV12. Our previous study has showed that an EGFP fused V12CBD recombinant protein could recognize multiple strains, including staphylococci, streptococci and enterococci (Dong Q, et al., Construction of a chimeric lysin Ply187N-V12C with extended lytic activity against staphylococci and streptococci, Microbial biotechnology, 2015), and V12CBD can bind to *S. aureus* with an affinity close to *S. aureus* antibody (Liu J, et al., Study of the interactions between endolysin and bacterial peptidoglycan on *S. aureus* by dynamic force spectroscopy, Nanoscale, 2015). In the previous manuscript we found that V12CBD inhibited adhesion and invasion of *S. aureus* to A549 cells. To further examine the specificity of this inhibition effect, we tested the effects of V12CBD on adhesion of *Listeria monocytogenes* (*Lmo*), *Salmonella enteritidis* (*Sen*) and *Streptococcus agalactiae* (*Sag*) to A549 cells under the same condition. Results showed that V12CBD has no significant effects on the adhesion of these three strains to A549 cells, implying that V12CBD could specifically inhibit the adhesion of *S. aureus* to A549 cells. These results have been added in the revised manuscript as **Fig 1c** (pages 6, lines 126-129).

Referee #2:
This study investigates the capacity of a cell-wall binding domain fragment from a phage lysin to alter the host response and subsequent virulence of Staphylococcus aureus. The recombinant protein was capable of altering uptake of S. aureus into both epithelial and professional phagocytic cells. The lysin protein also altered expression of S. aureus genes, increased proinflammatory cytokine production in response to S. aureus and reduced mortality in a murine model of infection (using two different strains of S. aureus). There are currently no vaccines or no non-antibiotic treatments approved for use against S. aureus so novel treatment options are highly desired. The development of a lysin/recombinant protein approach is novel. There are concerns on the mechanism behind the lysin that require attention that need to be addressed and thus strengthen the validity of the claims in the study.

Major concerns
A major concern revolves around the action of the lysin protein. It has been shown that the lysin is lytic to S. aureus. The authors need to demonstrate that the CBD domain of the protein used in this

study is not lytic, so that the mechanism explored in the study are not due to S. aureus being killed. All the data could be easily attributed to lysed bacteria.
Response: To exclude the lytic activity of V12CBD to *S. aureus*, we studied the effects of V12CBD on the growth rate and morphology of *S. aureus*. The optical density observation showed that 100 and 300 μg/ml V12CBD has minor effect on the growth rate of *S. aureus* T23. The thin-section electron microscopy further showed that rare changes in morphology were observed for *S. aureus* T23 after exposure to 100 and 300 μg/ml V12CBD for 1 h. These results showed that V12CBD could not lysis *S. aureus*. We added these additional data to the revised manuscript as **Appendix Fig S1** (page 6, lines 116-117).

First section of results, untreated vs treated. Could the result be just due to residual protein left on epithelial cells?
Response: As your concern, we labeled V12CBD with Alexa Fluor 488 and cocultured it with epithelial A549 cells for 24 h. The confocal macroscopy images showed that V12CBD could not non-specifically bind to, nor penetrate into A549 cells, implying a rare possibility for residual V12CBD left on the epithelial cells. These results have been added in the revised manuscript as **Appendix Fig S4**.

In figure 1 it is shown that the CBD influences both adhesion and invasion. Could the data from the invasion aspect be just a direct correlation to the adhesion data. There are probably less intracellular bacteria as a consequence of less bacteria adhering and being taken up by the cell.
Response: Yes, we agree with the above deduction. It is highly likely that less intracellular bacteria is the consequence of less bacteria adhering and being taken up by the cell. We added some discussion on this in the revised manuscript (page 19, lines 397-398).

The inhibitory capacity of CBD was also examined with macrophages. Is CBD capable of permeating cell membrane or being taken up by cells. Showing that CBD does not permeate the cell will demonstrate it is interfering with the phagocytic process and is not interfering with a function inside the cell.
Response: To test the cell penetrability of V12CBD, we labeled it with Alexa Fluor 488 and cocultured the labeled V12CBD with A549 and RAW264.7 cells for 1 and/or 24 h, respectively. Results showed that V12CBD could not permeate A549 cells, but can be taken up by macrophages in a time-dependent manner. These results have been presented as **Fig 3d** and **Appendix Fig S4** in the revised manuscript (page 11, lines 238-240). Our initial data showed increased expressions of inflammatory cytokines in macrophages after exposure to V12CBD for 24 h. However, we feel it difficult for us to design some further experiments to show whether the function inside the macrophages has been affected by V12CBD after it is taken up by the cell.

Signaling in response to S. aureus in the presence/absence of V12CBD was tested with polymyxin B to prevent signaling as a result of contaminating LPS. This could alternatively be done using LPS from Rhodbacter sphaeroides. The control experiment showing that polymyxin B is working (preventing signaling from LPS) is missing.
Response: We thank the suggestion of using LPS from *Rhodbacter sphaeroides* to antagonize *Escherichia coli* lipopolysaccharide. Since LPS from *Rhodbacter sphaeroides* was not ready available in our lab, we did additional control experiments to show that polymyxin B can completely block the stimulation effect of LPS from *E. coli* O55:B5 in the revised manuscript as **Appendix Fig S5** (page 12, lines 248-252). It also shows that expression levels of *Il1b*, *Il6*, *Tnfa* and *iNos* in macrophage RAW264.7 after exposure to 50 μg/ml V12CBD for 24 h in the presence or absence of 10 μg/ml polymyxin B had no much difference.

The point was made with signaling in response to S. aureus and V12CBD that it was NF-kB dependent. However, non NF-kB dependent genes were quantified.
Response: We tested many genes besides NF-κB dependent genes in our initial studies since we did not have the clues on the mechanism of V12CBD. In order to make the manuscript more concise, the qRT-PCR detection of the expression levels of non NF-κB dependent genes such as FIZZ1, MR and Arg1 **(previous Appendix Fig S5),** is deleted in the revised manuscript.

Minor concerns
Page 4 in the introduction, it is noted there are no antibody-based therapies for S. aureus. Some reference to the work being done with alpha toxin which is in phase 2/3 clinical trials should be

noted.
Response: These progresses have been added in the revised manuscript (page 4, lines 80-82) and reference (Hua L, Hilliard JJ, Shi Y, Tkaczyk C, Cheng LI, Yu X, Datta V, Ren S, Feng H, Zinsou R, Keller A, O'Day T, Du Q, Cheng L, Damschroder M, Robbie G, Suzich J, Stover CK, Sellman BR (2014) Assessment of an anti-alpha-toxin monoclonal antibody for prevention and treatment of *Staphylococcus aureus*-induced pneumonia. *Antimicrobial agents and chemotherapy* **58:** 1108-1117.)

Fig. 5b and f the data should be ordered the same way in each panel
Response: The original Fig 5b and 5f have been redrawn in the revised manuscript as **Fig 6b and 6f.**

Several spelling and grammatical errors were noted. The paper would benefit from editing by a native English speaker.
Response: The spelling and grammar have been checked again carefully in the revised manuscript.

Referee #3:
In this manuscript, Yang et al. demonstrate that treatment of Staphylococcus aureus with a recombinant protein derived from the cell wall binding domain of phage lysin PlyV12 (V12CBD) results in reduced virulence gene expression, reduced invasion of epithelial cells and increased susceptibility to killing by macrophages. Incubating macrophages with V12CBD leads to their activation and enhanced phagocytic activity. Furthermore, authors show that both therapeutic as well as prophylactic administration of V12CBD protects mice from systemic S. aureus infection. The subject of this paper is of high importance, and the authors report very interesting findings. Unfortunately, some sections of the manuscript are poorly written and would benefit from careful editing by a native speaker. Furthermore, authors often fail to explain their figures and ought to be more careful with their data interpretation (e.g Fig 5d-e). Thus, there are some major concerns the authors need to address.

Major concerns:
• Some further detail on the strains used would be suggested. Please reference them in Materials & Methods (N315 PMID:11418146 and T23).
Response: These information has been added in the *Materials & Methods* section of the revised manuscript (page 21, line 444).

• P4 Line 86 contains incorrect citations. These papers referenced here are not on S. aureus vaccines.
Response: These references have been replaced with (Fowler & Proctor, 2014; Jansen et al, 2013; Proctor, 2012) (Pages 4-5, lines 88-89).

• Figure 1c: What is the blue versus red line? This is not mentioned in main text nor in figure legend. I would suggest labeling graphs as Fig 1c) example FACS plot and Fig 1d) summary data of phagocytosis
Response: The Fig 1c and Fig 1d have been revised as suggested (now **Fig. 1d and 1e** in the revised manuscript). And the meanings of the cyan and red lines in the FACS plot have been added in the revised figure legend (page 30, line 649-650).

• Figure 1g: Which strain was used for infection? Main text says T23, while Figure legend says N315.
Response: It is T23 but not N315. This mistake has been corrected in the revised figure legend of **Fig 1h** (page 28, line 657).

• Figure 3d: greatest effect is seen with 100ug V12CBD. Why do authors choose to look at 25 and 50ug treatment?
Response: As you noted, in the macrophage killing assay, we pretreated the RAW264.7 cells with 0, 25, 50 and 100 μg/ml V12CBD, and found that treated with 100 μg/ml V12CBD resulted in the highest phagocytosis and the lowest survival of *S. aureus*. Because qRT-PCR is very sensitive, we choose a relatively low V12CBD concentration to see its effects on the expressions of *Il1b*, *Il6*, *Tnfa* and *iNos* in macrophages (now **Fig 3e** in the revised manuscript).

• *Figure 3f: please provide example FACS blot. What is the positive control here? Why do authors choose to treat with 100ug V12CBD here but with 25-50ug in Fig 3d?*
Response: The example FACS blots have been added in the revised manuscript and list as Fig 4a and 4c. The information of positive control used in the detection of CCR7, CD80, CD86 and MHCII was 15 ng/mL IFN-γ and 15 ng/mL LPS, while, for the detection of MR, 20 ng/mL IL-4 was used as the positive control. All the information has been added in Figure 4 and the figure legend in the revised manuscript. In the original Fig 3f, we used a V12CBD concentration of 50 μg/ml, but not 100 μg/ml, for all the flow cytometry detections. In the original Fig 3d (now **Fig 3e**), as explained above, because qRT-PCR is very sensitive, we choose a relatively low V12CBD concentration to see its effects on the expressions of *Il1b*, *Il6*, *Tnfa* and *iNos* in macrophages.

• *Figure 3g: Signaling inhibitors depicted in graph are neither mentioned in figure legend nor explained in text.*
Response: The information has been added in the legend of **Fig 4e** in the revised manuscript (page 32, lines 701-703).

• *It would make more sense if Supplemental Fig 3 would proceed Supplemental Fig 4 in main text as well as in order of figures.*
Response: Thanks for the suggestion. The text has been modified accordingly (page 12, lines 248-258). Furthermore, the original Fig S3 has been redrawn to include the new data of additional control experiments to show that polymyxin B can completely block the stimulation effect of LPS from *E. coli* O55:B5 in the revised manuscript as **Appendix Fig S5a** (page 12, lines 251-252). It also shows that expression levels of *Il1b*, *Il6*, *Tnfa* and *iNos* in macrophage RAW264.7 after exposure to 50 μg/ml V12CBD for 24 h in the presence or absence of 10 μg/ml polymyxin B had no much difference (**Appendix Fig S5b**).

• *Supplemental Fig 6: NFkB detection is not visible in this graph. Please change X-axis to log scale.*
Response: The X-axis has been changed to log scale as suggested and the figure has been shown as **Fig 4f** in the revised manuscript.

• *Figure 5b and Page 15, Line 320-322: since authors only used 10ul (a rather small volume) of 1ml organ homogenates to determine bacterial loads some of their samples were negative for CFU. I would suggest the authors plate the whole organ after not detecting any bacteria in their assay.*
Response: Thanks for your suggestion. Since several organs were sampled simultaneously to show the relative bacterial loads, we did several dilutions and took an aliquot of 10 μl from each dilution for plating in order to make consistent comparison. From the results, one could see that this method could reflect the changes of bacterial loads in organs after treatment with V12CBD. Therefore, some negative for CFU results after V12CBD treatment would not affect the conclusion that V12CBD treatment could reduce the bacterial loads. Actually we referred to the method described by Chen F *et. al.* (small-molecule targeting of a diapophytoene desaturase inhibits *S. aureus* virulence, Nature chemical biology, 2016) when recovering the cfu from organs. In our future experiments, we will follow the suggestion to plate the whole organ after not detecting any bacteria when using this method for CFU recovering.

• *Figure 5d and Page 15, Line 327: Text says TNFa levels are reduced after V12CBD treatment however graph shows an increase in TNFa (open circles) after V12CBD treatment. Did authors look at any other pro-inflammatory cytokines besides TNFa?*
Response: Thanks for pointing this error. The open circles showing a higher level of TNF-α should be PBS treated group, but not V12CBD treated group. This mistake has been corrected in the revised manuscript (now **Fig 6d**). As for the mice sera, we only detected the expression level of *TNF-α* cytokine. However, we added another experiment showing the expression levels of *Il1b, Il6, iNos and Tnfa* in V12CBD-treated macrophage RAW264.7 during co-culturing with *S. aureus* N315 for 0-24 h. Increased expressions of genes encoding IL-1β, IL-6 or TNF-α were observed in V12CBD treated macrophages after coculture with S. aureus for 4 and 8 h. However, decreased expressions of genes encoding IL-1β and TNF-α were observed in V12CBD treated macrophage after co-culture with *S. aureus* for 24 h, suggesting that V12CBD treated macrophages might have an alleviated inflammation after 24 h of *S. aureus* infection. These results have been added in the revised manuscript as **Appendix Fig S7** (pages 12-13, lines 263-271).

• Figure 5e and Page 15-16, Line 329-331: Since there is clearly increased production of TNFa the conclusion that there is less inflammatory injury in organs because of reduced TNFa levels does not make any sense. Please elaborate on histology images. What are we looking at besides that these are kidney and liver sections?
Response: As what we explained above, the original Fig 5d presented a contradictory meaning due to our error, that is, the open circles should be PBS, but not V12CBD. Therefore, the conclusion in the main text that V12CBD treated mice produced reduced level of TNF-α and thus may suffer from less inflammatory injury in organs were stand. In order to show the differences more clearly, we moved the histology images to the Supplementary Material section as **Appendix Fig S11** in the revised manuscript to show in larger scale. We labeled histology images of PBS treated mice tissue with arrows, which show obvious inflammatory in tissue sections. Alleviated injury could be seen in V12CBD pre-treated tissue sections, showing reduced interstitial inflammatory cell infiltration and dilation, and ameliorated histopathological changes by comparison with that of PBS pre-treated controls. This description is also added in main text (Page 16, line 347-350).

• Fig 5g) Please show weight changes post infection as % weight loss over time
Response: The original Fig 5g has been revised as suggested in the revised manuscript as **Fig 6e**.

• Page 14-15, Line 308-309: Authors should not conclude from a cytotoxicity testing in vitro to safety in vivo, instead should test if a high concentration of V12CBD in vivo has an adverse effect in mice.
Response: Following your suggestion, we tested the effects of a single high dose of 1 mg/mouse V12CBD on the morbidity and viability of injected mice. No harmful effects were observed within 10 days compared with that of PBS treated group. These results have been presented as **Appendix Fig S9** in the revised manuscript (page 15, lines 323-325).

3rd Editorial Decision 3rd May 2018

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three referees and their comments are provided below.

The referees appreciate that the analysis has been strengthened, but have some remaining issues that I would like to ask you to address in a revised version. Referee #3 has a few minor text edits while referee #1 request the inclusion of an additional control. I have looked at everything and I do agree with referee #1 that this is an important control that I would like to ask you to address.

You can use the link below to upload the revised version.

Looking forward to seeing the revised manuscript

REFEREE REPORTS

Referee #1:

In the original review from referee # 1 two major points were raised
1) that an E.coli contaminate could be responsible for the NfKb induced activity. This possibility is now acknowledged by the authors.
2) This second point stated: "We know nothing about the biophysical properties of the V12CBD protein. Could the effects on S. aureus attachment and invasion of A459 cells be non-specific? A proper control could be a similar segment of a phage (with similar charge and hydrophobic properties) that does not target S. aureus. Such a control protein should be expressed and purified the same way as the test substance." The authors ignore this in my mind critical control. They do however tell us the the protein/peptide has an very high IP (9.9) which sets the stage for non-specific charge effects. (That the peptide/protein dose not affect cell invasion by other bacteria does not exclude that the effect on S. aureus is non specific). A unrelated protein/peptide with similar biophysical properties should be included as a control to show specificity before I can recommend publication of this story.

Referee #2:

In this revised manuscript the authors have conducted several new and control experiments in order to address the reviewers' concerns. The authors have conducted sufficient studies to address comments made and to better substantiate their claims. The revised manuscript is recommended for publication.

Referee #3:

In this manuscript, Yang et al. demonstrate that treatment of Staphylococcus aureus with a recombinant protein derived from the cell wall binding domain of phage lysin PlyV12 (V12CBD) results in reduced virulence gene expression, reduced invasion of epithelial cells and increased susceptibility to killing by macrophages. Incubating macrophages with V12CBD leads to their activation and enhanced phagocytic activity. Furthermore, authors show that both therapeutic as well as prophylactic administration of V12CBD protects mice from systemic S. aureus infection. The subject of this paper is of high importance, and the authors report very interesting findings. The point by point letter by the authors is very satisfactory as they have addressed reviewers concerns in detail. Additional experiments have been performed and included into the revised manuscript, where it seemed appropriate and needed.

Minor criticism:
• The manuscript would still benefit from editing by a native English speaker.
• Just for consistency: please label Figure 4b 'IFNγ+LPS' instead of 'Positive', as authors did in Figure 4d.

Revision - authors' response 21st May 2018

Response to reviewers' comments

Referee #1:
In the original review from referee # 1 two major points were raised
1) that an E. coli contaminate could be responsible for the NfKb induced activity. This possibility is now acknowledged by the authors.
2) This second point stated: "We know nothing about the biophysical properties of the V12CBD protein. Could the effects on S. aureus attachment and invasion of A459 cells be non-specific? A proper control could be a similar segment of a phage (with similar charge and hydrophobic properties) that does not target S. aureus. Such a control protein should be expressed and purified the same way as the test substance." The authors ignore this in my mind critical control. They do however tell us the the protein/peptide has an very high IP (9.9) which sets the stage for non-specific charge effects. (That the peptide/protein dose not affect cell invasion by other bacteria does not exclude that the effect on S. aureus is non specific). A unrelated protein/peptide with similar biophysical properties should be included as a control to show specificity before I can recommend publication of this story.
Response: Following the suggestion, we expressed the CBD of *Listeria* phage lysin Ply511 (CBD511), which does not bind with *S. aureus* and has similar charge and hydrophobic properties to V12CBD (MW_{V12CBD} =18.4 kDa, pI_{V12CBD} = 9.96, Z_{V12CBD}=+14.1; MW_{CBD511} =15.5 kDa, pI_{CBD511} = 9.96, Z_{CBD511} = +13.9). CBD511 was also purified using the same way as V12CBD. It was found that CBD511 did not affect the adhesion and internalization of *S. aureus* N315, but did reduce that of *Listeria monocytogenes* (*Lmo*). These new data are presented as Appendix Fig S2 in the revised manuscript (pages 6-7, lines 129-133), which further proved that binding with V12CBD specifically suppresses adhesion and invasion of *S. aureus* to the epithelial cells.

Referee #2:

In this revised manuscript the authors have conducted several new and control experiments in order to address the reviewers' concerns. The authors have conducted sufficient studies to address

comments made and to better substantiate their claims. The revised manuscript is recommended for publication.
Response: Thanks for your comments.

Referee #3:

In this manuscript, Yang et al. demonstrate that treatment of Staphylococcus aureus with a recombinant protein derived from the cell wall binding domain of phage lysin PlyV12 (V12CBD) results in reduced virulence gene expression, reduced invasion of epithelial cells and increased susceptibility to killing by macrophages. Incubating macrophages with V12CBD leads to their activation and enhanced phagocytic activity. Furthermore, authors show that both therapeutic as well as prophylactic administration of V12CBD protects mice from systemic S. aureus infection. The subject of this paper is of high importance, and the authors report very interesting findings. The point by point letter by the authors is very satisfactory as they have addressed reviewers concerns in detail. Additional experiments have been performed and included into the revised manuscript, where it seemed appropriate and needed.

Minor criticism:
• The manuscript would still benefit from editing by a native English speaker.
Response: The manuscript has been checked thoroughly again and some grammar errors were corrected.

• Just for consistency: please label Figure 4b 'IFNγ+LPS' instead of 'Positive', as authors did in Figure 4d.
Response: The original Fig 4b has been modified to show the detailed information of positive controls instead of “positive” in the revised manuscript. Because there are two kinds of positive controls: 20 ng/mL IL-4 is used as the positive control of the detection of MR, and a mixture of 15 ng/mL IFN-γ and 15 ng/mL LPS is used as the positive control for the detection of CCR7, we labeled these positive controls separately in the revised Fig 4b.

Accepted 12th June 2018

Thank you for submitting your revised manuscript to The EMBO Journal. The revision has now been seen by referee #1 and as you can see from the comments below the referee appreciates the added control. I am therefore very pleased to let you know that we will accept the manuscript for publication here. Before sending you the final accept letter there are just a few things to sort out. I have provided a revision link below so that you can upload the changes. Once we get the revision in then I will send you the formal accept letter.

- I have sent the manuscript to our publisher for them to do their pre-publication check. They will send me back their comments tomorrow and I will pass it on to you so that you can incorporate their suggestions at this stage.

- I see that most of the M&M section is in the appendix. I would be good to have key parts in the main MS file. OK to have some in the appendix, but I would like to see at least the essential elements in the main file.

- The RNA seq should be deposited in a database and the accession number should be provided in the main manuscript file.

Referee #1:

I have read through the manuscript and agree that the additional control experiment now makes it suitable for publication.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yuhong Li and Hongping Wei
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2017-98045R2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were performed over multiple biological replicates. The sample sizes were determined based on general description in previous references to meet requirements for statistical analysis.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomly assigned to groups by the investigators independently.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes,the methods used in data analysis was described in the methods section and figure legends.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
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http://datadryad.org

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http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Suppliers for the antibodies used were provided in the Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Yes, available information was described in the Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes, this information was described in the Methods section.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Data Aavilability section is complete.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All the transcriptome data were presented in the Supplementary Materials as Appendix Table S1.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA