

Peer Review Information

Journal: Nature Microbiology

Manuscript Title: Rapid pathogen-specific recruitment of immune effector cells in the skin by secreted toxins

Corresponding author name(s): Michael Otto

Reviewer Comments & Decisions:

Decision Letter, initial version:
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Dear Dr. Otto,

Thank you for your patience while your manuscript "Non-classical fast immune response to pathogen invasion" was under peer review at Nature Microbiology. It has now been seen by our referees, whose expertise and comments you will find at the end of this email. In the light of their advice, we have decided that we cannot offer to publish your manuscript in Nature Microbiology.

From the reports, you will see that while reviewers find your work interesting, they raise concerns about the strength of the novel conclusions that can be drawn at this stage. In particular, they raise several concerns regarding the contribution of classical immune-signalling (referees #1), the signal and tissue specificity of leukocyte recruitment by PSMs (referee #2), the characterization of the infection site and technical improvement and interpretation of results from intravital images in leukocyte influx assays and adoptive transfer experiments (referee #3). Given the length of time that it would likely take to address these concerns thoroughly, these criticisms are sufficiently important as to preclude further consideration of your work in Nature Microbiology and suggest that your best option is to submit the manuscript in its current form to another journal.

However, if you do feel that you would be able to include additional work to address these points, we would be willing to consider an appeal, although please note that we would reassess novelty with respect to existing literature at the time of appeal and would be unlikely to trouble the referee again unless we felt that his/her concerns had been satisfied in full. In the case of a successful appeal and eventual publication, the received date would be that of the revised paper.

I am sorry that we cannot be more positive on this occasion, but hope that you find the referees' comments helpful when preparing your paper for resubmission elsewhere.

Yours sincerely,

{ redacted }

Reviewer Expertise:

Referee #1: Staphylococcus pathogenesis

Referee #2: Staphylococcus pathogenesis

Referee #3: Immune cell recruitment and imaging

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

The rapid detection and destruction of invading pathogens is essential for host immune defence. The prevailing model is that host immune cells in the tissues identify invading pathogens via pattern recognition receptors (PRR) that detect pathogen-associated molecular patterns (PAMPs). This leads to the secretion of chemoattractant cytokines that recruit immune cells such as neutrophils to the site of infection.

In this manuscript, Nguyen et al., demonstrate that neutrophils can directly detect the presence of *S. aureus* via the PSM group of staphylococcal toxins. This results in significantly faster recruitment of neutrophils to the infection site than was seen for classical immune-signalling mediated neutrophil recruitment.

This is an excellent piece of work, that represents an important advance in our understanding via well-designed experiments, clearly presented data and a clear and easy to follow text.

However, I feel that there is one question outstanding. Although the authors clearly show that bacteria expressing PSMs are required for rapid neutrophil recruitment, it is unclear if classical immune-signalling contributes to this process. Therefore, it would be hugely informative to repeat the experiment in figure 1 using synthetic PSMa3 compared with scrambled peptide. Fluorescent latex beads or similar could be used to mark the injection site. This would demonstrate that PSM alone is sufficient to drive rapid neutrophil recruitment.

Reviewer #2 (Remarks to the Author):

The manuscript entitled, "Non-classical fast immune response to pathogen invasion" by Nguyen et al describes a previously unrecognized early pathway of neutrophil recruitment to a cutaneous site of *S. aureus* infection through the action of PSM peptides. Utilizing in vivo imaging, the authors characterize temporal differences in neutrophil influx in response to WT *S. aureus* or a PSM deficient isogenic mutant revealing that the lack of PSM production blunts the cellular response within the first 3-6 hours post-infection. The PSM-induced response was mapped to the host EGR pathway, which was confirmed to be relevant through a series of elegant adoptive transfer studies utilizing labelled neutrophils from mice harboring genetic lesions in the PSM receptor (FPR) and EGR. The studies highlight a novel role for a bacterial toxin in cellular recruitment that is independent of canonical host signaling pathways, and suggest alternate targeting approaches to modulate infection that have temporal specificity. As

such, they are of broad interest. The studies are rigorously performed and documented with appropriate statistical analyses throughout. Several comments are noted below to enhance the communication of the findings and address important scientific questions relevant to the authors conclusions.

Major points:

1. Additional mechanistic insights on the in vivo expression and tissue diffusion of PSMs is needed to understand whether the findings described are specifically relevant in the context of the ear or more broadly relevant to pathogenesis. What is the time course for tissue expression of the PSMs in the ear model? Is this distinct from commonly used skin infection models? The ear is an extremely thin and well-vascularized epithelium, rendering it easy to conceptualize how toxin diffusion may impact cells that are within the vasculature. An examination of whether early leukocyte recruitment by PSMs (dependent on EGR signaling) is similarly affected in a distinct epithelial infection model such as backskin infection is essential to understand whether the findings in this study are broadly applicable to the epithelium or exhibit tissue specificity.
2. Does skin infection of EGR^{-/-} mice phenocopy the clinical findings of mice infected with PSM-deficient *S. aureus* (lesion size, tissue injury)? If this pathway is essential for the PSM-elicited neutrophil response, clinical infection outcome analysis will be important to present to solidify the role of this novel pathway in modulation of host immunity. A demonstration that EGR^{-/-} neutrophils exhibit intact chemotactic responses to distinct/canonical stimuli is important to illustrate the specificity of the PSM-EGR signal and ensure that these cells do not exhibit a more generalized migration defect.
3. It would seem that an early influx of neutrophils to the site of infection would curb infection. Additional insight on the relevance of early recruitment and PSM-mediated cellular injury of neutrophils would be beneficial. Are these cells recruited to be killed? Do the recruited neutrophils exhibit cellular death or PSM-mediated injury?

Minor points:

1. Line 90 – awkward wording ‘in the used chimeric dsRed mice’
2. The schematic in Figure 4 suggests that the PSMs may traverse the endothelium and interact with neutrophils. Is there existing data to support this model? It seems more likely that PSMs would act within the tissue space.

Reviewer #3 (Remarks to the Author):

In the present manuscript, the authors addressed their hypothesis that bacterial toxins are responsible for the fast recruitment of circulating leukocytes to the site of infection. They assessed this using a skin infection model in mice, as wild type and pms-deficient, mutant *S. aureus* were injected into the ear followed by intravital imaging of immune cell accumulation, where fewer leukocytes were recruited to the mutant *S. aureus*. In addition, the bacteria and toxins were added to human neutrophils, which were then investigated on the level of gene expression, and a toxin related overexpression of the transcriptional regulator Erg1 was found. The PMS effect on ERG1 expression was reduced by a MEK/ERK cellular signaling pathway inhibitor. Finally, using adoptive transfer of WT, as well as EGR1-

and FPR2-KO bone marrow cells prior to bacteria infection, the mechanism of leukocyte recruitment was further investigated at 10h post infection. Despite an interesting theory, the results of the study do not adequately correspond to the conclusions drawn. Below are my main concerns:

First the authors set out to investigate how *S. Aureus* and the mutant PSM deficient *S. Aureus* recruit immune cells in the ear of mice. Immune cells were tracked by using chimeric mice that have undergone irradiation and bone marrow transfer, resulting in DsRed-producing bone marrow derived cells.

Major:

- I lack information about engraftment success of the individual mice, what were circulating counts prior to infection?
- Figure 1a shows that both bacterial strains remain at the same number as was injected, which indicates that the bacteria do not need psm for surviving at the site of infection. In parallel, these numbers also demonstrate that the strain are eliminated by the immune system at comparable levels during the first 12 h post-infection, which to me suggests that the impaired recruitment of immune cells demonstrated in fig 1b is completely uninteresting in terms of fighting the bacterial infection. To prove my conclusion wrong, the authors must demonstrate a difference in bacterial burden at the infected site and later time points should be included in the study. Further, regarding the enumeration of bacteria at 5 and 12h, is there any possibility that part of the bacteria were actually on the skin and not in the ear? Has this been controlled or prevented? If yes how? The count for the PBS sham infected mice is needed as control. Adjusting the CFU axis scale to make it readable would be appreciated, all data plot between 1.107 and 1.108. Linear scales are fine when data are not spreading over several logs, which seems to be the case.
- Figure 1b: The number of red spots per time point first increase for 1h then decrease before it finally increase again, where this second phase is slower for the delta-psm mutant and the signal eventually reaches zero for the mutant. Compared to other studies in the same model of infection, immune cell accumulation is reported much earlier compared to here, and I wonder why, as well as which cells that are recruited. Please add PBS injections to this graph Is the impaired pattern of immune cell recruitment to bacteria due to the chimeric mouse model? How did these exps compare to the exps in Fig 4 when plotted in the same graph? In addition, only the second phase is commented upon in the main text, whereas I wonder about the biphasic response to bacterial infection. Are different circulating immune cells recruited at these different time points? Do these cells express the red fluorescence to similar degree?

I think it may be misleading to connect data from different mice ($t=6h$). The number of mice followed in each group is not specified, and the recorded 2h observation at 24h post infection is never documented or discussed. Only representative data are shown, would it be possible to present a figure using either an estimator for the groups that includes data from all animals or individual series?

- According to the figure 1b the data collected from WT and delta-psm mutant look alike the first 3h after post infection, but the illustrating movie 1 shows a very different picture during this time window. Why?
- In figure 1c and the illustrating movie 2, a large part of the scrutinized area is totally red. How is it possible to monitor the number of red spot per time point in this situation? Why did bacteria become invisible soon after infection in figure 1c and related movies? It is difficult to understand what the recruited cells attracted to especially when sham injections are not demonstrated. Do the bacteria disseminate into the ear? In the movie 2 for the delta-psm infected mouse (between 6 and 8 hours), a huge number of red cells is visible in the upper right corner (even the right side) of the picture while almost no cells are on the left part. The area circled with the green dotted line is somehow between these 2 areas. What can explain this appearance of inflammation? Is the injection site always relevant

for observation and data collection? How does this correlate with the data from figure 1b where the presented values are close to zero at this time point?

The authors conclude that the secreted toxin psm is crucial for rapid recruitment of neutrophils to the infected site. Based on my comments above in addition to no characterization of the infected site in terms of resident immune cell activation and cytokine/chemokine production, I do not agree. Further, the effect of the psm deficiency on the bacteria is not investigated enough.

Minor:

- Page 3, line 86-87, remove the two first parts of this sentence, it is common knowledge that FACS do not allow for in vivo assessments

- Where are fig 1d referred to in the text?

- Is the toxin present in circulation?

They then investigate how human neutrophils respond to different *S. aureus* including the mutant on the level of gene expression. Neutrophils are known to store their effector proteins in their different granules, and to not require gene expression for their activation and execution of effector functions at the afflicted site. Thus, the rationale for these experiments is poor. Instead, the secretome of the treated neutrophils would have been interesting to investigate. In addition, the table inadequately explains the different experimental groups and treatments, and the reasoning and conclusions drawn are difficult to follow. It seems like the results claim that *S. Aureus* activates neutrophils solely by psm, which sounds unlikely. I would like the different bacterial strains to be characterized in respect to metabolites, production of PAMPs, other virulence factors etc. How do concentrations used here compare to the in vivo situation, how many bacteria are required to produce the amounts of psm?

The differences in gene expression were then investigated more closely, and differences in EGFR1 were found, despite that Fig 2 does not show any sign changes. Again, I question the rationale for these exps, and would have liked to see the protein levels.

Lastly, adoptive transfer experiments were performed to demonstrate the signaling important for *S. aureus*-induced immune cell recruitment at 10h post infection using conventional confocal exps.

- What happens to fluorescently labeled transferred leukocytes remains elusive, to what extent are they circulating, trapped in lung vasculature or dead and do the different genetic strains impact on the fate of these cells under non-infected conditions? How is the relative part of WT, ERG1^{-/-} and FPR2^{-/-} leukocyte in the total leukocyte population different from the population injected 12h before sacrifice (1:1:1) and why? Spleen does not quite correlate to peripheral circulation. Why is the spleen population more relevant than the circulating population or injected population? Can ERG1^{-/-} and FPR2^{-/-} cells have different retention time in the spleen compared to wild type?

- There are huge variations in the in vivo experiments using the different cells, what statistics are used to get significance in fig 4C?

- These exps do not discriminate between FPR deficiency in circulating neutrophils or tissue recruited bone marrow derived monocytes and macrophages.

- In this last part of the manuscript (figure 4), the cell count is done 10 hours post infection only and thus difficult to compare with the initial kinetic observation. Data at 10 hours post infections, acquired in the same condition, from mice from the fig1 would be helpful.

- Further, 10h post-infection is pretty long, what is the fate of labeled cells that traffic to the site of

infection, perhaps they undergo apoptosis and can no longer be visualized?

- In figure 4b there is more spleen measurement data than animals in 4c.
- About the normalization of cell counts, the transformation used is difficult to understand (lines 621 to 624). I think that showing increase or decrease in frequency when compared to the reference population would better represent a specific recruitment, rather than the normalized numbers presented now.
- What happens to the bacterial burden at the site of infection? These expts are crucial to demonstrate importance of the hypothesis.
- Finally, it has been reported that serum lipoprotein particles are responsible for the binding and inhibition of psm toxins and that the toxin is produced by *S. aureus* upon phagocytosis by neutrophils. It would be interesting to monitor the local and circulating toxin concentrations.

Author Rebuttal to Initial comments

Reviewers Comments:

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[Reply: Thank you very much for these encouraging comments! As a response to criticism by other reviewers, we have completely redone the intravital imaging experiment shown in Fig. 1 using another](#)

technique (essentially using another mouse strain in which leukocytes and particularly neutrophils have bright fluorescence). This allowed us to perform more repeats and gather more precise influx data for statistical analysis. The obtained data completely confirm our conclusions and as we think further considerably strengthen our message. Using this new setup, we also included the experiment the reviewer asked for. We used injection of PSM α 3 in comparison to a PSM α 3 derivative (K12A) of which we previously had shown strongly reduced pro-inflammatory activity. We thought this to be a better control than a completely scrambled peptide. Please see **Fig. 1c** for results. The derivative of PSM α 3 did not cause leukocyte influx, with values reduced to those obtained with a PBS-only control.

In addition to a large number of further experiments that we performed for this revision mostly due to criticism by other reviewers (see list above), we also would like to draw the reviewer's attention to **Fig. 2d**, where we now measured activation of WT, EGR1^{-/-}, and FPR2^{-/-} neutrophils (Ca²⁺ flux) in response to PSM α 3 and the PSM α 3K12A derivative, demonstrating EGR1- and FPR2-dependence of neutrophil activation by PSM α 3 but not the PSM α 3K12A derivative control.

Reviewer #2 (Remarks to the Author):

The manuscript entitled, "Non-classical fast immune response to pathogen invasion" by Nguyen et al describes a previously unrecognized early pathway of neutrophil recruitment to a cutaneous site of *S. aureus* infection through the action of PSM peptides. Utilizing in vivo imaging, the authors characterize temporal differences in neutrophil influx in response to WT *S. aureus* or a PSM deficient isogenic mutant revealing that the lack of PSM production blunts the cellular response within the first 3-6 hours post-infection. The PSM-induced response was mapped to the host EGR pathway, which was confirmed to be relevant through a series of elegant adoptive transfer studies utilizing labelled neutrophils from mice harboring genetic lesions in the PSM receptor (FPR) and EGR. The studies highlight a novel role for a bacterial toxin in cellular recruitment that is independent of canonical host signaling pathways, and suggest alternate targeting approaches to modulate infection that have temporal specificity. As such, they are of broad interest. The studies are rigorously performed and documented with appropriate statistical analyses throughout. Several comments are noted below to enhance the communication of the findings and address important scientific questions relevant to the authors conclusions.

Major points:

1. Additional mechanistic insights on the in vivo expression and tissue diffusion of PSMs is needed to understand whether the findings described are specifically relevant in the context of the ear or more broadly relevant to pathogenesis. What is the time course for tissue expression of the PSMs in the ear model? Is this distinct from commonly used skin infection models? The ear is an extremely thin and well-vascularized epithelium, rendering it easy to conceptualize how toxin diffusion may impact cells that are within the vasculature. An examination of whether early leukocyte recruitment by PSMs (dependent on EGR signaling) is similarly affected in a distinct epithelial infection model such as backskin infection is

essential to understand whether the findings in this study are broadly applicable to the epithelium or exhibit tissue specificity.

Reply: Thank you for these questions. As for the time course of PSM expression: We now measured PSM expression during the time frame of the ear infection model and later on. The data are shown in **Supplementary Fig. 1b**. These data show substantial expression of PSM genes particularly during early hours of infection. We also compared to expression kinetics in a back skin infection, which were similar. Furthermore, our observations indicate that the extent of abscess formation in the ears that is dependent on PSMs (as judged from the comparison of WT and Δpsm infected mice) was very similar to that which we generally observe at the back of mice. However, we only observed this initially in some mice and because abscesses soon grow larger than the size of mouse ears, measuring abscess formation in the ears could not be adequately determined and would have been unethical. Therefore, it was measured in the back skin infection model – the common site to perform such a model.

As for the generalizability of ear infection model data, we are afraid that the type of imaging we performed is not possible at other sites, like the back of the mouse, as the imaging depends on the fact that the ears are thin. This is why the ear is commonly used for this type of experiment. However, we now included in our study also a back skin infection model, which cannot be used for imaging, but with which we demonstrate that EGR1 has a significant impact on *S. aureus* infection control. This we think is an important addition to our manuscript, demonstrating the relevance of the signaling mechanism that we report for infection control. We hope that our new experiments answer at least to some extent, within what is technically possible, to the concerns of the reviewer.

2. Does skin infection of EGR^{-/-} mice phenocopy the clinical findings of mice infected with PSM-deficient *S. aureus* (lesion size, tissue injury)? If this pathway is essential for the PSM-elicited neutrophil response, clinical infection outcome analysis will be important to present to solidify the role of this novel pathway in modulation of host immunity. A demonstration that EGR^{-/-} neutrophils exhibit intact chemotactic responses to distinct/canonical stimuli is important to illustrate the specificity of the PSM-EGR signal and ensure that these cells do not exhibit a more generalized migration defect.

Reply:

To answer the reviewer's question, we need to remind the reviewer that PSMs are toxins that have a strong and often reported major impact on skin infection (e.g. Wang et al. Nat Med 2007; Nakagawa et al. Cell Host Microbe 2017; Li et al. Sci Rep 2016). (We believe this to be the very reason that the host has developed the sensing mechanism we describe.) A PSM-negative mutant therefore shows a strong deficiency in causing abscesses. An EGR1^{-/-} mutant, as it would be deficient in the pathway to react to those toxins, is expected to show a larger abscess, rather than phenocopy that of a PSM-deficient mutant. We now included a longer-term back skin abscess model to directly analyze the impact of EGR1 on

infection control (see **Fig. 5**). We believe this data, which shows much larger abscesses in EGR1^{-/-} than WT mice, to represent a strong addition to our manuscript, as it shows the relevance of EGR1 for infection control. The fact that this effect is absent in mice infected with a PSM-deficient mutant links it to PSMs. Of note, to analyze these effects, we had to reduce the inoculum to a level at which the PSM toxin-dependent impact in WT bacteria became low and hardly significant, as the abscesses in the EGR1^{-/-} mice at higher inocula became too big.

Furthermore, we now present data on neutrophil stimulation by fMLP, a “canonical” secreted stimulus, which show unaltered responses in EGR1^{-/-} neutrophils.

3. It would seem that an early influx of neutrophils to the site of infection would curb infection. Additional insight on the relevance of early recruitment and PSM-mediated cellular injury of neutrophils would be beneficial. Are these cells recruited to be killed? Do the recruited neutrophils exhibit cellular death or PSM-mediated injury?

Reply: There are several papers that have reported on the toxin-related biological aspects of PSMs. Our paper focused on PSM-mediated signaling. The abovementioned data in the back skin infection model that we now obtained demonstrate biological relevance of the sensing mechanism for infection control. We hope the reviewer agrees that further examination of PSM toxin-mediated fate of leukocytes *in vivo* is beyond the scope of the present paper, which already contains a wide array of experimental data from several different angles.

Minor points:

1. Line 90 – awkward wording ‘in the used chimeric dsRed mice’

Reply: We don’t use these mice anymore (and thus this sentence was deleted), as the experiment shown in Fig. 1 was completely redone.

2. The schematic in Figure 4 suggests that the PSMs may traverse the endothelium and interact with neutrophils. Is there existing data to support this model? It seems more likely that PSMs would act within the tissue space.

Reply: Unfortunately, at the concentrations PSMs are active as signaling molecules, they cannot be detected in tissue or blood (by HPLC or antibodies). Please note, however, that it was the very purpose of the adoptive transfer experiment to answer the question whether or not PSMs act in the tissue space via resident cells in regard to the reported mechanism of EGR1 activation. Our data (**Fig. 4**) show that resident cells are not involved, which has the direct implication on leukocyte recruitment from the blood

as depicted in the scheme (now **Fig. 5c**). As the reviewer alludes to, we also considered this to be unlikely before we performed our experiments, which in our opinion strengthens the significance and novelty of the mechanism we report.

Reviewer #3 (Remarks to the Author):

In the present manuscript, the authors addressed their hypothesis that bacterial toxins are responsible for the fast recruitment of circulating leukocytes to the site of infection. They assessed this using a skin infection model in mice, as wild type and pms-deficient, mutant *S. aureus* were injected into the ear followed by intravital imaging of immune cell accumulation, where fewer leukocytes were recruited to the mutant *S. aureus*. In addition, the bacteria and toxins were added to human neutrophils, which were then investigated on the level of gene expression, and a toxin related overexpression of the transcriptional regulator *Erg1* was found. The PMS effect on *ERG1* expression was reduced by a MEK/ERK cellular signaling pathway inhibitor. Finally, using adoptive transfer of WT, as well as *EGR1*- and *FPR2*-KO bone marrow cells prior to bacteria infection, the mechanism of leukocyte recruitment was further investigated at 10h post infection. Despite an interesting theory, the results of the study do not adequately correspond to the conclusions drawn. Below are my main concerns:

Reply: We performed a large series of experiments for this revision, which are listed at the beginning of this document, and which we hope answer to the concerns of the reviewer. We also would like to add that our manuscript generally and especially after this revision contains much effort to provide detailed controls that in similar form are not often provided, for example the PBS control for skin infection the reviewer asked for, or the direct analysis of actually injected CFU in our animal models combining results from different infected mice.

First the authors set out to investigate how *S. Aureus* and the mutant PSM deficient *S. Aureus* recruit immune cells in the ear of mice. Immune cells were tracked by using chimeric mice that have undergone irradiation and bone marrow transfer, resulting in DsRed-producing bone marrow derived cells.

Major:

- I lack information about engraftment success of the individual mice, what were circulating counts prior to infection?

Reply: We now completely redid the experiment shown in Fig. 1, predominantly as a response to this reviewer's criticism, not using DsRed mice and engraftment anymore, but *C57BL/6-Lysozyme^{tm1}M-GFP* mice. The new setup allowed for better reproducibility, statistical analysis, distinction of neutrophils and other leukocytes, avoidance of strong saturation effects, and better visualization of bacteria. This was a

major and laborious experimental endeavor, with only one mouse at a day undergoing intravital imaging at our central facility.

- Figure 1a shows that both bacterial strains remain at the same number as was injected, which indicates that the bacteria do not need psm for surviving at the site of infection. In parallel, these numbers also demonstrate that the strain are eliminated by the immune system at comparable levels during the first 12 h post-infection, which to me suggests that the impaired recruitment of immune cells demonstrated in fig 1b is completely uninteresting in terms of fighting the bacterial infection. To prove my conclusion wrong, the authors must demonstrate a difference in bacterial burden at the infected site and later time points should be included in the study.

Reply: Our influx model was set up to analyze initial events after infection, the major focus of our study, rather than the ultimate impact of the discovered mechanism on infection outcome. The analysis of bacterial CFU in that context was meant to ascertain that there are yet no strong effects of bacterial elimination or outgrowth that would have made our assessment of chemotactic attraction of leukocytes difficult to interpret.

However, we absolutely agree with the reviewer that the impact on infection outcome was an important issue to address and therefore now included a back skin infection model that demonstrates an impact of EGR1 on infection outcome (Fig. 5a,b). It also shows PSM dependence to the degree that is technically possible, considering that PSMs have a well-characterized opposite impact on skin infection, which we believe to be the very reason for host defense having developed a mechanism to sense these molecules as signals for bacterial invasion.

Further, regarding the enumeration of bacteria at 5 and 12h, is there any possibility that part of the bacteria were actually on the skin and not in the ear? Has this been controlled or prevented? If yes how? The count for the PBS sham infected mice is needed as control.

Reply: We now included, as requested, a control with PBS. There are virtually no contaminating bacteria from the skin, demonstrating the efficiency of the ethanol sterilization step that we performed for the very purpose of eliminating skin-colonizing bacteria.

Adjusting the CFU axis scale to make it readable would be appreciated, all data plot between 1.10^7 and 1.10^8 . Linear scales are fine when data are not spreading over several logs, which seems to be the case.

Reply: We now changed this graph to a linear y axis, as requested. Furthermore, the graph now contains the results from PBS sham-infected mice, also as requested. It was moved to Supplementary Material (Supplementary Fig. 1a), as the completely redone in-vivo influx imaging experiment and the PSM peptide/PSM peptide derivative data using that model (requested by reviewer 1) filled up Fig. 1.

• Figure 1b: The number of red spots per time point first increase for 1h then decrease before it finally increase again, where this second phase is slower for the delta-psm mutant and the signal eventually reaches zero for the mutant. Compared to other studies in the same model of infection, immune cell accumulation is reported much earlier compared to here, and I wonder why, as well as which cells that are recruited. Please add PBS injections to this graph Is the impaired pattern of immune cell recruitment to bacteria due to the chimeric mouse model? How did these exps compare to the exps in Fig 4 when plotted in the same graph? In addition, only the second phase is commented upon in the main text, whereas I wonder about the biphasic response to bacterial infection. Are different circulating immune cells recruited at these different time points? Do these cells express the red fluorescence to similar degree? I think it may be misleading to connect data from different mice (t=6h). The number of mice followed in each group is not specified, and the recorded 2h observation at 24h post infection is never documented or discussed. Only representative data are shown, would it be possible to present a figure using either an estimator for the groups that includes data from all animals or individual series?

Reply: Many of the concerns the reviewer had are now answered because the experiment shown in **Fig. 1** was completely redone using a different setup.

As for further concerns:

- Early accumulation of immune cells/biphasic response: As our new data show, the minor very early (1-2 h p.i.) increase in signal in our previous data, which the reviewer referred to, were a technical problem rather than true influx. These problems were now overcome by use of a different setup. We believe the influx kinetics (significant influx starting at 4 – 6 h p.i.) are similar to other reports in the literature and did not differ – except for that initial blip - between the previous and the new data. The new setup generally allowed for more reproducibility and also to much greater extent avoided saturation effects toward later time points. Due to saturation effects the previous data had suggested that the difference in influx between WT and Δpsm infected mice starts to disappear earlier than what the new data show. The effect is thus actually more pronounced and longer-lasting, emphasizing and strengthening our central message.
- Different immune cells: This question of the reviewer was related to the two phases of the response, which as we explain were due to a technical issue. The new data do not show a biphasic response. The reviewer previously also agreed with us that flow cytometry cannot be used in this setting, which is why we are happy that the new detection method allowed in a previously reported way to distinguish neutrophils from other leukocytes, giving at least some information on this specific leukocyte type that is generally considered the most important in the early response to infection, also better linking our data to the whole-genome gene expression data that were performed with neutrophils.

- According to the figure 1b the data collected from WT and delta-psm mutant look alike the first 3h after post infection, but the illustrating movie 1 shows a very different picture during this time window. Why?
- In figure 1c and the illustrating movie 2, a large part of the scrutinized area is totally red. How is it possible to monitor the number of red spot per time point in this situation? Why did bacteria become invisible soon after infection in figure 1c and related movies? It is difficult to understand what the recruited cells attracted to especially when sham injections are not demonstrated. Do the bacteria disseminate into the ear? In the movie 2 for the delta-psm infected mouse (between 6 and 8 hours), a huge number of red cells is visible in the upper right corner (even the right side) of the picture while almost no cells are on the left part. The area circled with the green dotted line is somehow between these 2 areas. What can explain this appearance of inflammation? Is the injection site always relevant for observation and data collection? How does this correlate with the data from figure 1b where the presented values are close to zero at this time point?

Reply: All these concerns should be addressed by the fact that we now used a completely new setup for the experiment shown in Fig. 1.

The authors conclude that the secreted toxin psm is crucial for rapid recruitment of neutrophils to the infected site. Based on my comments above in addition to no characterization of the infected site in terms of resident immune cell activation and cytokine/chemokine production, I do not agree. Further, the effect of the psm deficiency on the bacteria is not investigated enough.

Reply: We believe that with the new experimental additions/changes to our manuscript we have provided ample and sufficient evidence for PSM-mediated rapid recruitment of neutrophils to the infection site. Using isogenic *psm* mutants we have shown clear effects on neutrophil recruitment in vivo (Fig. 1). Furthermore, please also consider that we performed the experiments shown in Fig. 4 directly to address the question of involvement of resident immune cells, which those results clearly rule out.

As for the effect of PSM deficiency on the bacteria, this has been investigated in several studies. On the transcriptional level, there are only minor changes that mostly include transcription of the PSM exporter, *Pmt* (Joo et al. MBio 2016). There are no known effects on other potentially pro-inflammatory molecules. We previously also showed that PSMs can shed lipoproteins from the cell surface, which act via TLR2 (Hanzelmann et al. Nat Commun 2016). We now address this in the text and also used pure PSMs to elicit the rapid neutrophil attraction response (as suggested by reviewer 1) in lines 110-118. These results strongly suggest a mechanism underlying the shown PSM-mediated effects that is directly exerted by PSMs.

Finally, we understand that the reviewer finds it surprising that in our in-vitro gene expression (microarray) studies, PSMs showed a dramatic effect that indicates a negligible role of any other secreted

pro-inflammatory factors in the investigated phenotype. We have explained in the text that this is in fact not surprising given the hugely higher abundance of PSMs as compared to any other secreted (and cell surface-released) pro-inflammatory factors (such as other toxins). Naturally, in-vitro results can be influenced by the growth conditions of the culture filtrates used (growth time, media). This is exactly why we further analyzed the role of PSMs and the identified EGR1 signaling axis in vivo (Fig. 4), confirming that role of PSMs. Please note that while we demonstrate a strong impact of PSMs on rapid neutrophil recruitment in vivo, nowhere in our manuscript do we claim exclusivity in-vivo as for PSMs being the only factors contributing to that phenotype.

Minor:

- Page 3, line 86-87, remove the two first parts of this sentence, it is common knowledge that FACS do not allow for in vivo assessments

Reply: In our considerably changed manuscript, we do not state this anymore.

- Where are fig 1d referred to in the text?

Reply: This entire section has been changed.

- Is the toxin present in circulation?

Reply: We definitely would have loved to show presence of PSMs in circulation (or in tissues), not only for this study but generally in our work. PSMs are pro-inflammatory in low concentrations, at which detection in the context of body fluids is simply impossible. We tried this often and using different approaches. We commonly use HPLC to detect PSMs, which works well with bacterial media where there are higher concentrations, but not for lower concentrations and in that background, where many other factors co-elute in the elution range (even after concentration using butanol extraction). We also produced antibodies against PSMs, and while they are fairly specific, they are by far not sensitive enough for such low concentrations.

However, we believe that our results using isogenic mutants and ko mice in the adoptive transfer experiments clearly show dependence of PSMs and rule out involvement of resident tissue cells, suggesting that PSMs attract neutrophils directly by diffusion into the blood.

They then investigate how human neutrophils respond to different *S. aureus* including the mutant on the level of gene expression. Neutrophils are known to store their effector proteins in their different granules, and to not require gene expression for their activation and execution of effector functions at the afflicted

site. Thus, the rationale for these experiments is poor. Instead, the secretome of the treated neutrophils would have been interesting to investigate.

Reply: The reviewer may to a certain extent have misunderstood our intentions. Our aim was to investigate the mechanism underlying fast neutrophil recruitment and not neutrophil killing mechanisms, which the reviewer adequately describes as due to release from granules of stored factors. For this purpose, analysis of transcriptional responses made more sense than analysis of the protein secretome. We also note that by a secretome analysis we would not have found the crucial role of EGR1 in that response, which represents a key message of our manuscript.

In addition, the table inadequately explains the different experimental groups and treatments, and the reasoning and conclusions drawn are difficult to follow.

Reply: We would like to draw the reviewer's attention to the fact that the microarray experiment was a huge experiment including multiple conditions, to the point where we could not even upload the Excel sheet with the raw data to the journal's website. For that reason we referred to the GEO website where those data are accessible in their entirety, and we encourage the reviewer to check those.

Our study contains a series of approaches and experimental data, of which this extended microarray experiment only represents one. We therefore had to find a way to present and discuss all the data and conclusions from the microarray experiment in a condensed fashion. We have tried to make some things clearer in this revision while also keeping the respective text as short as possible. We have thought for a long time on how to present the data from the microarray experiment, and on which to focus, so that they fit and comprise those that are most important for our manuscript. We are happy to introduce specific changes upon request.

It seems like the results claim that *S. Aureus* activates neutrophils solely by psm, which sounds unlikely. I would like the different bacterial strains to be characterized in respect to metabolites, production of PAMPs, other virulence factors etc. How do concentrations used here compare to the in vivo situation, how many bacteria are required to produce the amounts of psm?

Reply: We do not believe that it is "unlikely" that PSMs play a major role among secreted *S. aureus* factors in stimulating neutrophil gene expression. We have explained this in our manuscript: Note that according to our hypothesis of the role of secreted factors in early induction of neutrophil gene expression, we focused on secreted (cell surface-released) factors, while most other pro-inflammatory

factors are surface-anchored. Furthermore, PSMs are present in the culture filtrate in amounts vastly exceeding those of other secreted pro-inflammatory factors, such as other toxins (see our previous publications, for example Wang et al. Nat Med 2007, and those from other groups ever since). As we already stated above, the in-vitro conditions we used may to a certain extent impact the relative contribution of different factors to the phenotype under investigation, but first, we used frequently used growth conditions (TSB, 8 h growth), and second, we confirmed the major role of PSMs in neutrophil attraction in vivo. Finally, as also already stated above, we do not claim exclusivity of PSMs being the only secreted factors attracting neutrophils, we merely state that under the used conditions they have a dominant impact, and we believe the in-vitro and in-vivo results in that regard being straightforward and clear.

The differences in gene expression were then investigated more closely, and differences in EGFR1 were found, despite that Fig 2 does not show any sign changes. Again, I question the rationale for these exps, and would have liked to see the protein levels.

Reply: As already explained above, we believe that when looking for signaling affecting rapid responses, assaying transcriptional changes makes more sense. This sort of analysis led us to a major result of our paper, which a secretome analysis would not have.

As for significance in Fig. 2, we note that these are all results from the microarray analysis that have been arranged in graphs to compare and illustrate the most important changes and comparisons. This is sometimes done with color scales, but this would not have illustrated in an adequate fashion the dramatic changes we observed. Please note that all these values are the result of a sophisticated analysis of significance routinely performed for microarray analyses, as described in methods, and only significant changes are shown.

Lastly, adoptive transfer experiments were performed to demonstrate the signaling important for *S. aureus*-induced immune cell recruitment at 10h post infection using conventional confocal exps.

- What happens to fluorescently labeled transferred leukocytes remains elusive, to what extent are they circulating, trapped in lung vasculature or dead and do the different genetic strains impact on the fate of these cells under non-infected conditions?

Reply: The presence of the fluorescently labeled transferred leukocytes in the spleens and the recruitment of these cells into the infected ears demonstrated that these cells were capable of circulating in the vasculature as well as generally able to reach tissues and organs. Quantification and analysis of the number of labeled WT, FPR2^{-/-}, and EGR1^{-/-} cells, which were adoptively transferred at equal proportions, in the spleens of the infected mice revealed that the differences in genetic composition, namely deficiency of EGR1 and FPR2 compared to WT, did not impact survival of these cells. That this would be different in non-infected animals is highly unlikely, as there is already no difference in infected animals. Moreover,

the infection site is distant and the infection is minor and only local. We believe our spleen data already provide for more control data than often provided in similar experiments. We believe measurement of infiltration into more organs is not of much further value or common for our purpose which was to measure influx to the site of infection.

How is the relative part of WT, ERG1^{-/-} and FPR2^{-/-} leukocyte in the total leukocyte population different from the population injected 12h before sacrifice (1:1:1) and why?

Reply: We are not sure what exactly the reviewer means here. Probably, the reviewer refers to the difference in absolute numbers, while the 1:1:1 ratio was maintained (?). This is due to the setup of the experiment maximizing the injected numbers of cells and matched-pair analysis. We maximized the number of cells that was adoptively transferred in each experiment (every mouse) by adjusting every 1:1:1 mix to the leukocyte preparation from bone marrow with the lowest yield (WT, EGR1^{-/-}, or FPR2^{-/-}). We apologize that this had not been explained sufficiently in the initial version of our manuscript. It is now much better explained in the manuscript text:

“Note that the absolute numbers of leukocytes varied in the 1:1:1 mixes, and each adoptive transfer experiment represents a separate experiment in a different mouse, which were combined in the data shown in panels **b-g**. Therefore, influx numbers show wide variation between experiments. This was addressed by using matched data (repeated measures) analyses.”

Furthermore, we changed the graphical presentation to include lines between matching data points to illustrate this analysis better.

Spleen does not quite correlate to peripheral circulation. Why is the spleen population more relevant than the circulating population or injected population? Can ERG1^{-/-} and FPR2^{-/-} cells have different retention time in the spleen compared to wild type?

Reply: We used the spleen controls to analyze whether 1) cells are circulating 2) genetic differences impact survival and tissue infiltration. We chose the spleen because it is well-established as (and the largest) secondary lymphoid tissue. We believe measurement in either circulation or the spleens can be used as valuable control in our experiment, and they both have pros and cons. As stated above, that we performed such controls already exceeds what is done in many other studies with similar experiments of infiltration to an infection site. We also note that our data were not much changed overall by using the spleen controls, and the same sort of significant changes we report would have been observed without that normalization.

- There are huge variations in the in vivo experiments using the different cells, what statistics are used to get significance in fig 4C?

Reply: We believe this criticism refers to the same situation we explained above. The yield of the leukocyte isolation varied, and we wanted to use as many leukocytes as possible, adjusting to that sort of leukocyte (WT, FPR2^{-/-}, EGR1^{-/-}) in the 1:1:1 mix that gave the lowest yield. This variation was addressed statistically by using a matched pair analyses. We now explain this better in the figure legend:

“Note that the absolute numbers of leukocytes varied in the 1:1:1 mixes, and each adoptive transfer experiment represents a separate experiment in a different mouse, which were combined in the data shown in panels **b-g**. Therefore, influx numbers show wide variation between experiments. This was addressed by using matched data (repeated measures) analyses.”

Furthermore, we changed the graphical presentation to include lines between matching data points to illustrate this analysis better.

- These exps do not discriminate between FPR deficiency in circulating neutrophils or tissue recruited bone marrow derived monocytes and macrophages.

Reply: Correct. These experiments cannot distinguish between which sort of leukocyte is attracted, which was not our intention. However, we note that we have now included in the results in Fig. 1 an analysis of neutrophils versus other leukocytes, showing that – as expected from the literature – neutrophils are the main type of leukocytes attracted by PSMs in our model.

- In this last part of the manuscript (figure 4), the cell count is done 10 hours post infection only and thus difficult to compare with the initial kinetic observation. Data at 10 hours post infections, acquired in the same condition, from mice from the fig1 would be helpful.

Reply: As the experiment in Fig. 1 was completely redone, there is now direct comparison at 10 h. Also note that the new results in Fig. 1 using a new setup, which does not show saturation of the detection as early as in our previous setup, show that 10 h is well situated in the time range where a strong PSM effect is apparent.

- Further, 10h post-infection is pretty long, what is the fate of labeled cells that traffic to the site of infection, perhaps they undergo apoptosis and can no longer be visualized?

Reply: If there was differential apoptosis of leukocytes dependent on their FPR2 or EGR1 status, this would be controlled for by the spleen controls.

- In figure 4b there is more spleen measurement data than animals in 4c.

Reply: We would like to thank the reviewer for this constructive comment and recognize that the graphical presentation of the data was confusing. To this end, we have replotted, organized, and presented the data in a more straightforward manner.

To explain the number of shown data points: First, we sometimes used both ears for WT (LAC) bacteria injection, which was the case with these data for 2 mice (\Rightarrow 4 data points), and later only one ear per mouse, in this case for 3 mice (\Rightarrow 3 data points) (\Rightarrow total of 7 data points in panels c,d, except for one group, where there are only 6 because one ear injection failed.). Therefore, there are spleen data from 5 mice. Second, there are three differently labeled donor leukocyte types, meaning 3 data points/mouse \Rightarrow 15 data points in total/group. Because the purpose of this graph was to show that there are no significant influx differences into the spleen dependent on the genotype of the transferred (donor) cells, we show spleen values that are sorted by donor and grouped together for all recipient mice (WT, EGR1^{-/-}, FPR2^{-/-}) (rather than separated by recipient versus donor type as in the ear influx data shown in panels c,d and Supplementary Figure 6.) In the previous version, we did not distinguish the different recipient genotypes in the spleen data graph by color as we did now, and we hope this coloring makes the analysis more easily understandable. We also adjusted the figure legend with explaining text.

In the case of the *psm* deletion strain – data now shown in Fig. 4e-g and the corresponding supplemental data, we always used one ear per mouse, so that we also have 5 mice in total, and 15 total data points for the spleen values, again distinguishing recipients by coloring.

- About the normalization of cell counts, the transformation used is difficult to understand (lines 621 to 624). I think that showing increase or decrease in frequency when compared to the reference population would better represent a specific recruitment, rather than the normalized numbers presented now.

Reply: It sounds complicated but it really isn't: Let's say we found in the spleen a relation of 1 (WT) : 0.8 (FPR2) : 0.8 (EGR1) then we multiplied the ear FPR2 and EGR1 values by 1/0.8.

Comparing the frequencies as the reviewer suggests would not be much different, but have the disadvantage that we would have to do statistics on frequencies/percentages which is not entirely appropriate. We therefore kept the current form of analysis via normalization.

We should also add that we performed the spleen normalization to be very correct – overall, results would not have been much different without that normalization.

- What happens to the bacterial burden at the site of infection? These exps are crucial to demonstrate importance of the hypothesis.

Reply: We understand that the reviewer wanted a readout of whether the mechanism we describe is consequential for infection. We absolutely agree that this is important, which is why we added the experiment now shown in Fig. 5. We used as readout abscess size rather than CFU as we (and others) found that pathogenesis of *S. aureus* abscesses is better reflected by abscess measurement than CFU.

- Finally, it has been reported that serum lipoprotein particles are responsible for the binding and inhibition of psm toxins and that the toxin is produced by *S. aureus* upon phagocytosis by neutrophils. It would be interesting to monitor the local and circulating toxin concentrations.

Reply: We agree, but as we stated above, it is not technically possible to determine PSM concentrations at that level (pro-inflammatory concentrations) in the context of tissue/blood. We have tried HPLC/MS with/without an initial extraction step as well as specific antibodies, which are the most adequate approaches one can possibly take.

Decision Letter, first revision:

Dear Dr. Otto,

Thank you for submitting your revised manuscript "Non-classical fast immune response to *Staphylococcus aureus* skin invasion" (NMICROBIOL-20020439A-Z). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Sincerely,

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{redacted}

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Reviewer #1 (Remarks to the Author):

The authors have fully addressed my request for an additional experiment, resulting in data that greatly support their original conclusion.

I have no further points to raise.

Reviewer #2 (Remarks to the Author):

The revised study very nicely addressed the reviewer comments, and extended the findings to further support the novel conclusions of the study. My only recommendation is for the results section to comment on the potential for underlying genetic differences in the EGR-/- mice (B6N-129 background) compared to the controls (B6/B6N) to contribute in part to the differences observed in the infection outcome. The massive lesions in the EGR-/- mice certainly implicate this pathway, but the observation that infection with the PSM- strain elicits a lesion that is similar in size to WT *S. aureus* infection in the WT mice may be the result of two biological processes: 1) non-PSM contributors to the phenotype which is commented on, and 2) B6N-129 genetic differences that lead to increased infection susceptibility overall. This could only be directly assessed through the use of EGR+/+ or +/- mice on the B6N/129 background; given the overall strength of the data in support of a role of PSM in EGR-mediated host signaling, embarking upon this series of controls is not warranted, but should be at least mentioned as an experimental consideration.

Reviewer #3 (Remarks to the Author):

Thank you for your efforts to address my previous concerns. Congratulations to a well done study.

Decision Letter, final checks:

Dear Dr. Otto,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "Non-classical fast immune response to Staphylococcus aureus skin invasion" (NMICROBIOL-20020439A-Z). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: <https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication> for details).

In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Non-classical fast immune response to Staphylococcus aureus skin invasion". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

{redacted}

Reviewer #1:

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The authors have fully addressed my request for an additional experiment, resulting in data that greatly support their original conclusion.

I have no further points to raise.

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Final Decision Letter:

Dear Michael,

I am pleased to accept your Article "Rapid pathogen-specific recruitment of immune effector cells in the skin by secreted toxins" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

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