

T3SS effector EseJ in *Edwardsiella piscicida* inhibits PANoptosis in macrophages

Corresponding Author: Professor Hai Xia Xie

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Communications Biology, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The same set of Authors (many common authors) have published the article in Cellular Microbiology and somehow did not cite (given below)
Cell Microbiol, 2020 Jul;22(7):e13193. doi: 10.1111/cmi.13193. Epub 2020 Mar 5.
Edwardsiella piscicida type III protein EseJ suppresses apoptosis through down regulating type 1 fimbriae, which stimulate the cleavage of caspase-8, Tian Tian He 1 2, Ying Zhou 1 2, Ying Li Liu 1 2, Duan You Li 1 2, Pin Nie 1 3 4, Ai Hua Li 1, Hai Xia Xie,

This paper is extension with few more additions. However, why the previous paper is not cited in this manuscript?

Major Comments

1. Figure 9, the schematic is wrongly represented. All the arrows for apoptosis , pyroptosis etc are going up instead of going down. If the Authors are stating that EseJ inhibits Panoptosis, then all the form of death, arrows should go down in the presence of EseJ. In the absence of EseJ, the Panoptosis will go high.
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- 4.Cytokine ELISA just be done for the gasdermin activation
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In this manuscript, Tian Tian He and colleagues detail how the EseJ type III secretion effector from *Edwardsiella piscitida* inhibits PANoptosis. The data is convincing and thorough and should be of interest at least for all those studying host-pathogen interactions, in particular the role of effector proteins, and also for those interested in animal cell death pathways. The main weakness I notice is that almost all experiments are done in mice cells, while *E. piscitida* is a fish pathogen. Do the same EseJ mechanism of inhibition of PANoptosis applies in fishes?

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9. Lines 135-137, there is no reference to a figure. I also find it a bit difficult by looking at figures 1a or 2b to appreciate the cell rounding, cell swelling, and cell rupture of cells infected by the *eseJ* mutant strain. Maybe zoomed images or arrows could help.
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13. In Fig. 7, perhaps it would be easier to grasp if the legend shows the protein that is inhibited, instead of the chemical inhibitor.
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The Authors have answered all the queries in their revised manuscript

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Overall, the authors have adequately addressed my previous concerns.

However, I still have a few minor points that should be considered:

1. Line 37: The correct term here is virulence, which is a quantitative term (i.e., can be increased or decreased), while pathogenicity is a qualitative term.
2. Lines 191–192: I find this sentence somewhat confusing and repetitive relative to the introduction. Consider rephrasing to improve clarity, perhaps starting with "In previous studies..." or otherwise revising the introduction to this section.
3. Lines 203–206: The relevant detail is still difficult to visualize; I had to zoom in to 400% on my monitor. It may help to show the zoomed-in image directly in the "Cells" panel and to clearly indicate the zoomed regions in the original panels (for the 3 h time point). Consider adding a supplementary figure showing a wider field of the cells.
4. Lines 310–318: The description of the caspase-8 and NLRP3 knockdowns should be moved to the Results section rather than appearing in the Discussion. If possible, improve the quality of the immunoblot. Additionally, the statement beginning

“Strikingly... NLRP3 knockdown (Fig. S1)” should be toned down, as the current evidence does not fully justify such a strong conclusion. With the current data maybe a more general sentence like “the data support that inhibition of caspase-1 activation by EseJ depends on caspase-8 and NLRP3” would be better.

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This paper is extension with few more additions. However, why the previous paper is not cited in this manuscript ?

Response: This is a misconception. That paper has been quoted several times in the manuscript, please see lines 70, 121, 168, 325, 340, and 422 in the original submission. That paper primarily reported that EseJ suppresses caspase-8-mediated apoptosis by down-regulating type 1 fimbriae (He et al., 2020). In the current work, it was found that EseJ suppresses cell apoptosis in murine macrophages at an early stage after infection, followed by a suppression of pyroptotic cell death at a late time point. Furthermore, T3SS-translocated EseJ inhibits PANoptosis by activating TAK1. So there is a misunderstanding here. And this study is definitely not an addition to the previous publication, but goes further in exploring the new discovery.

Major Comments

1. Figure 9, the schematic is wrongly represented. All the arrows for apoptosis , pyroptosis etc are going up instead of going down. If the Authors are stating that EseJ inhibits Panoptosis, then all the form of death, arrows should go down in the presence of EseJ. In the absence of EseJ, the Panoptosis will go high.

Response: The fork at EseJ indicates that EseJ is depleted and therefore PANoptosis levels are increased. In the revision we have made the labeling easier to see in the schematic.

2. *In vivo* fish model (fig 8) showed a marginal difference in their survival index showing that the WT bacteria-infected fish showed the least survival but the mutants also showed reduced survival.

Response: At the early post-infection stage (day 3), the mortality rates in WT-infected fish are significantly higher than that of Δ eseJ-infected fish, suggesting that EseJ contributes to the virulence of *E. piscicida*. The Δ eseJ strain fails to replicate inside the host cell and induces a significant increase in PANoptosis, which could lead to rapid activation of the immune system, forcing clearance of the Δ eseJ strain. Instead, the WT strain could disrupt host immune signaling, evade host clearance, replicate and spread in the host, leading to mass mortality. Mortality rates between WT- and Δ eseJ-infected fish are similar in the late post-infection period. This may be due to the fact that T3SS plays a major role in the early phase of infection, whereas T6SS plays a major role in the late phase (Yin et al., 2018; Li et al., 2021).

References:

Yin K, Guan Y, Ma R, Wei L, Liu B, Liu X, Zhou X, Ma Y, Zhang Y, Waldor MK, Wang Q. 2018. Critical role for a promoter discriminator in RpoS control of virulence in *Edwardsiella piscicida*. PLoS Pathog 14(8):e1007272.

Li DY, Liu YL, Liao XJ, He TT, Sun SS, Nie P, Xie HX. 2021. Identification and characterization of EvpQ, a novel T6SS effector encoded on a mobile genetic element in *Edwardsiella piscicida*. Front Microbiol 12:643498.

3. *All the caspase blots must have total caspase staining. Actin is fine, but total caspase must be stained with antibody to total caspase 8 or caspase 3, as the case may be.*

Response: This has been solved in the revision, please see new Fig. 3a, new Fig. 3d and new Fig. 3e in our revision.

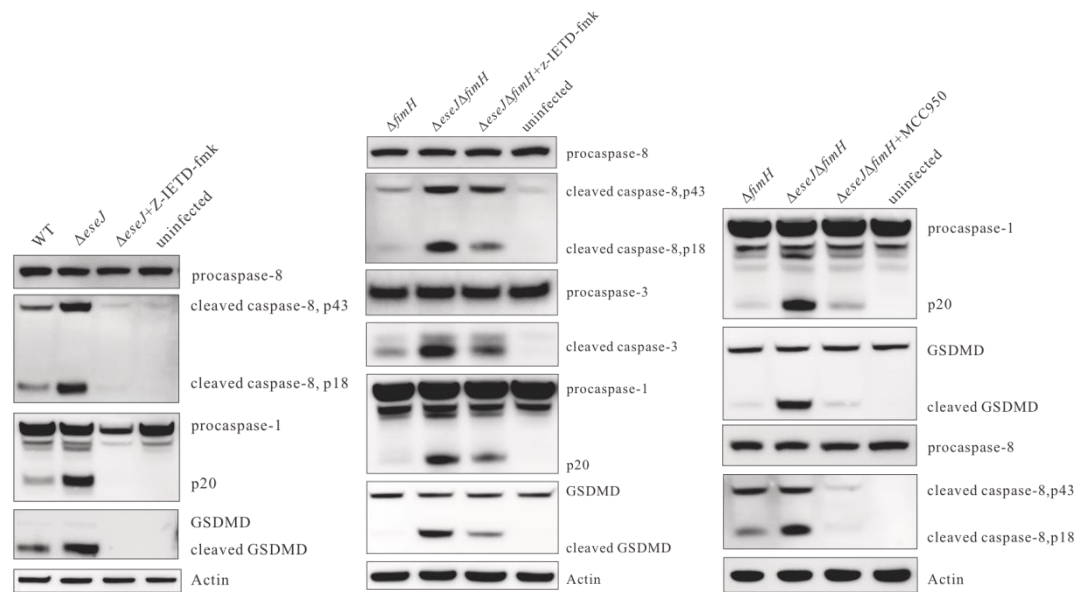


Figure legends:

Left panel: Immunoblotting of procaspase-8, cleaved caspase-8, cleaved caspase-1 and cleaved GSDMD from J774A.1 cells infected with *E. piscicida* WT or *ΔeseJ* strain at 3 hpi. J774A.1 cells were pretreated with the caspase-8 inhibitor Z-IETD-fmk for 1 h prior to infection with the *ΔeseJ* strain. Actin was used to confirm that similar amounts of protein were loaded per lane (new Fig. 3a).

Middle panel: Immunoblotting of procaspase-8, cleaved caspase-8, pro-caspase-3, cleaved caspase-3, cleaved caspase-1 and cleaved GSDMD from J774A.1 cells infected with *E. piscicida* *ΔfimH* or *ΔeseJΔfimH* strain at 3 hpi. J774A.1 cells were pretreated with the caspase-8 inhibitor Z-IETD-fmk for 1 h prior to infection with the *ΔeseJΔfimH* strain. Actin was used to confirm that similar amounts of protein were loaded per lane (new Fig. 3d).

Right panel: Immunoblotting of procaspase-8, cleaved caspase-8, cleaved caspase-1 and cleaved GSDMD from J774A.1 cells infected with *E. piscicida* *ΔfimH* or *ΔeseJΔfimH* strain at 3 hpi. J774A.1 cells were pre-treated with the NLRP3 inhibitor MCC950 for 1 h prior to infection with the *ΔeseJΔfimH* strain. Actin was used to confirm that similar amounts of protein were loaded per lane (new Fig. 3e).

4. Cytokine ELISA just be done for the gasdermin activation

Response: Very good suggestion. The release of the cytokines IL-1 β or IL-18 often follows the activation of caspase-1 and GSDMD. IL-1 β secretion levels were examined in J774A.1 cells infected with *E. piscicida* WT, *ΔeseJ*, and *ΔeseJ[eseJ]*

strains or *E. piscicida* $\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$, and $\Delta eseJ\Delta fimH$ strains. Please refer to the new Fig. 4o & 4p in our revision. Infection with the $\Delta eseJ$ strain stimulates a significant decrease in IL-1 β secretion. This is inconsistent with the observation that EseJ inhibits the activation of GSDMD and caspase-1. It is speculated that EseJ may play a role in the transcription or processing of pro-IL-1 β . This requires further study.

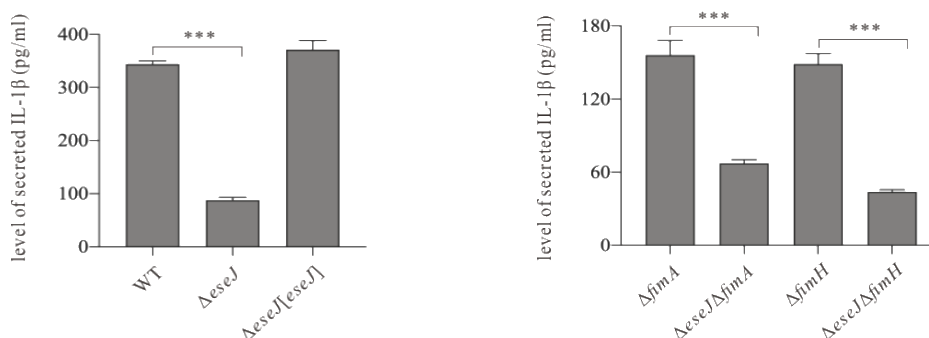


Figure legend: J774A.1 cells were infected with WT, $\Delta eseJ$, and $\Delta eseJ[eseJ]$ strains (Fig. 4o) or $\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$, and $\Delta eseJ\Delta fimH$ strains (Fig. 4p) for 5 h, after which culture supernatants were collected and the levels of IL-1 β secreted were assayed by ELISA. Supernatant from a non-infected cell culture was used as a control. Means $\pm$ SD of a representative experiment are shown. ***, $P < .001$.

5. With all the combinations of mutants and blocking caspases in macrophages, the bacterial burden must be measured. The CFU in macrophages under all different conditions and mutant constructs, with and without caspase inhibitors must be measured.

Response: Very good suggestion. The bacterial load in macrophages was examined at 3 h post infection in each experiment. The detailed results are outlined below:

Revised Fig. 2a-2g, 4a, 4b, 4i, and 4j: WT, $\Delta eseJ$, and $\Delta eseJ$ complemented with *eseJ* (via high-copy plasmid pACYC184 or low-copy plasmid pWSK29) strains (**Fig. a**);

Revised Fig. 2h-2k: WT, $\Delta eseJ$, $\Delta esaN$, and $\Delta eseJ\Delta esaN$ strains (**Fig. b**);

Revised Fig. 2l, 2m, 4c, 4d, 4k, and 4l: $\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains (**Fig. c**);

Revised Fig. 2n & 2o: WT, $\Delta eseJ$, $\Delta fimH$, and $\Delta fimH[eseJ]$ strains (**Fig. d**);

Revised Fig. 3a-3c: WT, $\Delta eseJ$, and $\Delta eseJ$ +Z-IETD-fmk (caspase-8 inhibitor) (**Fig. e**);

Revised Fig. 3d: $\Delta fimH$, $\Delta eseJ\Delta fimH$, and $\Delta eseJ\Delta fimH$ +Z-IETD-fmk (caspase-8 inhibitor) (**Fig. f**);

Revised Fig. 4e - 4g: $\Delta fimH$, $\Delta eseJ\Delta fimH$, $\Delta eseJ\Delta fimH$ +MCC950 (NLRP3 inhibitor) (**Fig. g**);

Revised Fig. 4m & 4n: WT, $\Delta eseJ$, WT+MCC950, and $\Delta eseJ$ +MCC950 (NLRP3 inhibitor)(**Fig. h**).

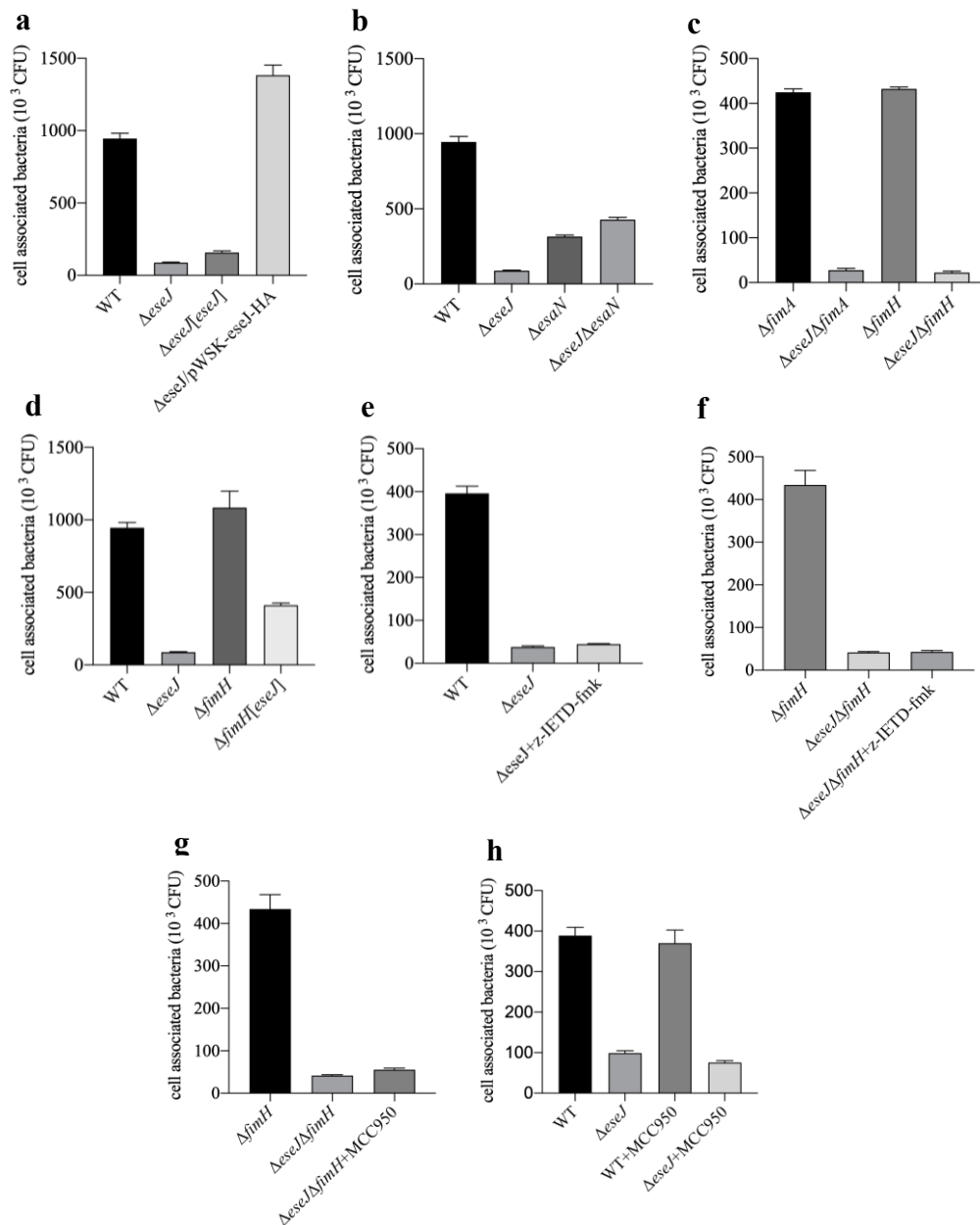


Figure legend: Quantification of cell-associated bacteria in J774A.1 cells infected with *E. piscicida* WT, $\Delta eseJ$, $\Delta eseJ[eseJ]$ and $\Delta eseJ/pWSK-eseJ-HA$ strains (**a**); or *E. piscicida* WT, $\Delta eseJ$, $\Delta esaN$ and $\Delta eseJ\Delta esaN$ strains at MOI of 10 (**b**); or *E. piscicida*

$\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains at MOI of 20 (c); or *E. piscicida* WT, $\Delta eseJ$, $\Delta fimH$ and $\Delta fimH[eseJ]$ strains (d); or J774A.1 cells were pre-treated with caspase-8 inhibitor Z-IETD-fmk for 1 h before infection with the $\Delta eseJ$ strain (e); or J774A.1 cells were pre-treated with caspase-8 inhibitor Z-IETD-fmk for 1 h before infection with the $\Delta eseJ\Delta fimH$ strain (f); or J774A.1 cells were pre-treated with NLRP3 inhibitor MCC950 for 1 h before infection with $\Delta eseJ\Delta fimH$ strain (g); or J774A.1 cells were pre-treated with NLRP3 inhibitor MCC950 for 1 h before infection with WT, and $\Delta eseJ$ strains (h).

Reviewer #2 (Remarks to the Author):

In this manuscript, Tian Tian He and colleagues detail how the EseJ type III secretion effector from Edwardsiella piscicida inhibits PANoptosis. The data is convincing and thorough and should be of interest at least for all those studying host-pathogen interactions, in particular the role of effector proteins, and also for those interested in animal cell death pathways. The main weakness I notice is that almost all experiments are done in mice cells, while E. piscicida is a fish pathogen.

Do the same EseJ mechanism of inhibition of PANoptosis applies in fishes?

Response: As suggested, fish leukocytes were isolated from head kidney of mandarin fish and infected with $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains to examine LDH release (see new **Fig. 3h** in revision). A significant increase in cytolytic cell death in leukocytes, one of the hallmarks of pyroptosis or PANoptosis, was found to be stimulated by further deletion of EseJ in the $\Delta fimH$ strain. Consistent with the observation in murine macrophages, pretreatment fish leukocytes with the inhibitor of caspase-8 and NLRP3 before infection with the $\Delta eseJ\Delta fimH$ strain decreased the release of LDH. The $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains were chosen for infection because of their similar levels of adhesion and invasion.

Major issues:

1. E. piscicida is a fish pathogen and all experiments are done with a mouse macrophage cell line. I suppose that few reagents would be available to analyse mechanistic details in fish cell lines. But at least cell death/PI staining? Are the inhibitors used specific to mammals ?

Response: As suggested, the fish leukocytes from head kidney were isolated and

pre-treated prior to infection with *E. piscicida* strains using the caspase-8 inhibitor Z-IETD-fmk or the NLRP3 inhibitor MCC950. As shown in new **Fig. 3h**, deletion of *EseJ* stimulated a significant increase in LDH release. However, pretreatment with Z-IETD-fmk or MCC950 prior to infection dramatically reduced LDH release. The results indicate that through the caspase-8 and NLRP3 signaling pathways, *EseJ* inhibits lytic cell death in fish leukocytes. This is similar to the observation in murine macrophages.

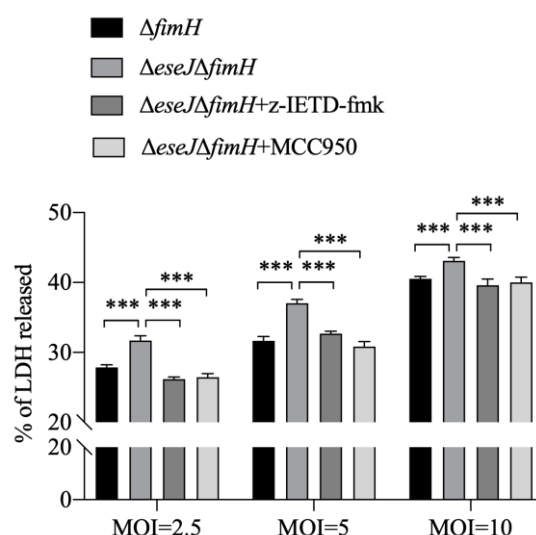


Figure legend: Fish leukocytes were treated with the caspase-8 inhibitor z-IETD-fmk or the NLRP3 inhibitor MCC950 for 1 h prior to infection with the $\Delta eseJ\Delta fimH$ strain at an MOI of 2.5, 5 or 10. Leukocytes infected with $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains were used as negative and positive controls, respectively. At 3 h post infection, culture supernatants were collected and the amount of LDH released was measured. ***, $P < .001$.

2. Figure 4e/f, there are many possible explanations (e.g., improper protein folding) that could explain why some truncated versions of *EseJ* function better than others. I suggest removing this data from the manuscript, as it also does not add much to the story.

Response: We agree. These two figures have been omitted in the revision.

3. Figure 6i, I suppose this was done at least twice. Showing the quantification of the immunoprecipitated caspase-8 and RIPK1 could support the authors conclusions.

Response: Very good suggestion. Quantification of immunoprecipitated caspase-8 and RIPK1 from three independent experiments is shown in new **Fig. 4m & 4n**. After

MCC950 pre-treatment, a significant decrease in immunoprecipitated caspase-8 and RIPK1 was detected in J774A.1 cells infected with the WT strain compared to the Δ *eseJ* strain.

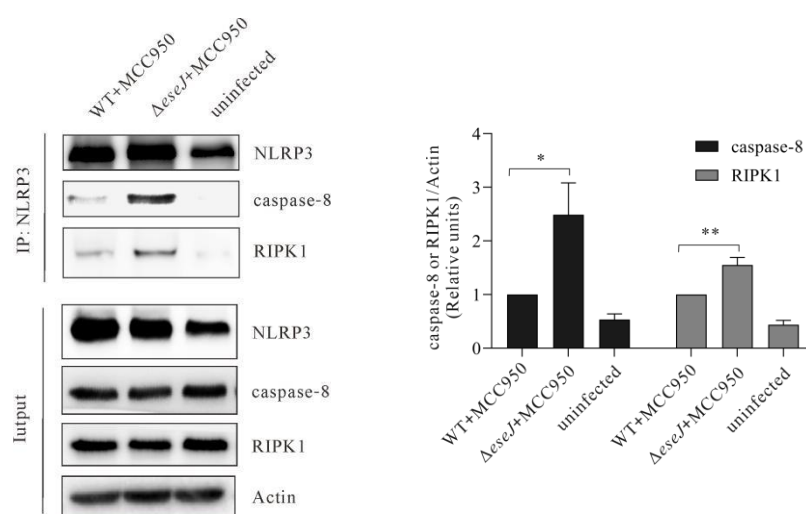


Figure legend

new Fig. 4m: Immunoblotting of the PANoptosome components RIPK1 and Caspase-8 in the input and in immunoprecipitation by the endogenous NLRP3 from lysates of J774A.1 cells which are pretreated with MCC950 prior to infection with WT and Δ *eseJ* strains.

new Fig. 4n: Quantitative analysis of caspase-8 and RIPK1 from immunoprecipitated samples shown in the left panel. The amount of caspase-8 or RIPK1 was quantified by densitometry and normalized to that of actin. The graph shows the relative ratios (Means $\pm$ SEM) of caspase-8 or RIPK1 from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

4. Most of the mechanistic data is based on the use of inhibitors. The authors should at least acknowledge this in their discussion as a limitation of the study. Key siRNA-mediated depletion or infection of cells from knock-out mice (MEFs) would make the conclusions stronger.

Response: Very helpful suggestion. We have acknowledged this limitation in the discussion, see lines 310 to 312. In fact, we have constructed plvx-*nlrp3*-siRNA and plvx-*caspase-8*-siRNA plasmids. These plasmids were packaged into lentiviruses and used to stably knock down NLRP3 and caspase-8 in J774A.1 cells. At 48 h post transduction, the cells were infected with Δ *fimH* or Δ *eseJ* Δ *fimH* strains at an MOI of

10. After 3 hours of infection, cells and supernatants were harvested and the expression levels of caspase-8, NLRP3, cleaved caspase-1 (p20) were examined by immunoblotting (Fig. S1). The *nlrp3* siRNA significantly reduced the protein level of NLRP3, indicating the knock down of NLRP3 in J774A.1 cells. Furthermore, transduction with *nlrp3* siRNA obviously reduced the level of cleaved caspase-1 (p20) in J774A.1 cells infected with the Δ *eseJ* Δ *fimH* strain. Caspase-8 siRNA treatment also reduced caspase-1 levels. These results indicate that the activation of caspase-1 stimulated by infection with the Δ *eseJ* Δ *fimH* strain is mediated by NLRP3 and caspase-8.

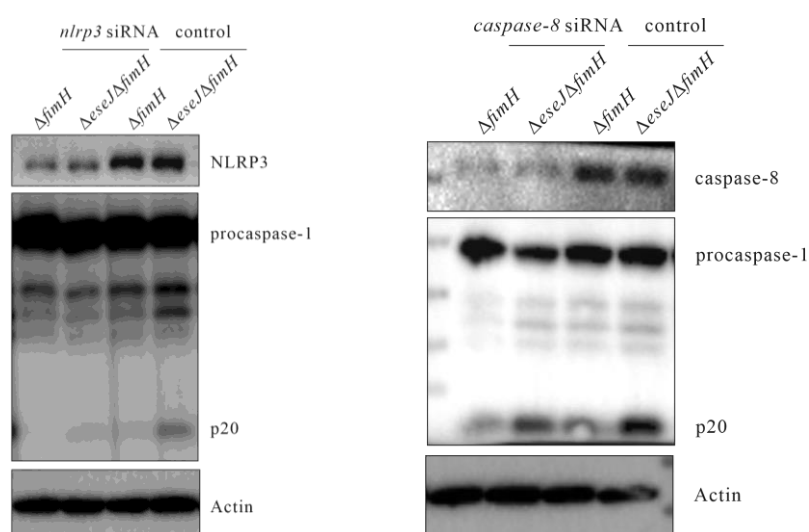


Figure legend: Immunoblotting of caspase-8, NLRP3, cleaved caspase-1 (p20) in J774A.1 cells transduced with plvx-*nlrp3*-siRNA (left panel) and plvx-*caspase-8*-siRNA (right panel) plasmids or plvx-control (non-sense sequence)-siRNA packaged into lentiviruses for 48 h prior to infection with Δ *fimH* or Δ *eseJ* Δ *fimH* strains at an MOI of 10 for 3 h. The supernatants and cell lysates were harvested and probed with different antibodies. Actin was used to confirm that similar amounts of protein were loaded per lane.

Other/minor issues:

1. The title has a significant number of acronyms. I do not have an alternative but suggest it should be revised.

Response: It has been revised as “T3SS effector EseJ in *Edwardsiella piscicida* inhibits PANoptosis in macrophages”

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Response: Corrected.

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Response: Corrected.

6. *Lines 81 and 82. Is this true for ALL pathogens?*

Response: Thank you for pointing this out. We have revised the sentence as "Many pathogens can stimulate one or more of these three PCD pathways during infection."

7. *Line 108, mechanisms is better than strategies (implying rational thinking, which bacteria do not have).*

Response: Corrected.

8. *Line 133, proteins do not have phenotypes; please revise the writing.*

Response: Corrected.

9. *Lines 135-137, there is no reference to a figure. I also find it a bit difficult by looking at figures 1 a or 2b to appreciate the cell rounding, cell swelling, and cell rupture of cells infected by the eseJ mutant strain. Maybe zoomed images or arrows could help.*

Response: It is Fig. 2a, this information has been added in the revision. In the new Fig. 3b, we have added annotated zoomed panels with arrows that explicitly highlight the Δ eseJ-induced morphological changes in J774A.1 cells - including cell rounding

at 1 hpi and cell swelling and rupture at 3 hpi.

10. Line 143, it seems full complementation by providing *eseJ* in trans; why "partially rescued?". For LDH release it does not seem so clear, but PI staining, yes.

Response: Yes, it is fully complementary for the LDH release, and on closer inspection, it is also fully complementary for the PI staining. This sentence has been rephrased in the revision.

11. Line 190, "delivered into host cells" would be better than "translocated into hosts".

Response: Corrected.

12. Figure 6i, typo in caspase 8.

Response: Corrected.

13. In Fig. 7, perhaps it would be easier to grasp if the legends hows the protein that is inhibited, instead of the chemical inhibitor.

Response: It has been changed as you suggested in the revision. This change makes it easier for the reader to follow the story.

14. It would make more sense to me if the story would start with the role of *EseJ* on virulence of *E. piscicida*, and then analyse how it works.

Response: Very helpful suggestion. The story begins with the role of *EseJ* in the virulence of *E. piscicida* in the revision.

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Reviewer #2 (Remarks to the Author):

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However, I still have a few minor points that should be considered:

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Response: Corrected.

2. Lines 191–192: I find this sentence somewhat confusing and repetitive relative to the introduction. Consider rephrasing to improve clarity, perhaps starting with "In previous studies..." or otherwise revising the introduction to this section.

Response: It has been rephrased as “In our previous study, we demonstrated that EseJ inhibits host cell apoptosis by specifically suppressing the type 1 fimbria-dependent activation of caspase-8 and following cleavage of caspase-3 (lines 206-207). ”

3. Lines 203–206: The relevant detail is still difficult to visualize; I had to zoom in to 400% on my monitor. It may help to show the zoomed-in image directly in the "Cells" panel and to clearly indicate the zoomed regions in the original panels (for the 3 h time point). Consider adding a supplementary figure showing a wider field of the cells.

Response: Very helpful suggestion. In the new Fig. 3b, we have added an annotated, zoomed-in image to the ‘Cells’ panel. This image uses arrows to highlight the Δ eseJ-induced morphological changes in J774A.1 cells, including cell rounding at 1 hpi and cell swelling and rupture at 3 hpi. Additionally, stitched panorama images were used to demonstrate wider-field views of Fig. 3b; these are provided in the

supplementary information.

4. Lines 310–318: The description of the caspase-8 and NLRP3 knockdowns should be moved to the Results section rather than appearing in the Discussion. If possible, improve the quality of the immunoblot. Additionally, the statement beginning “Strikingly... NLRP3 knockdown (Fig. S1)” should be toned down, as the current evidence does not fully justify such a strong conclusion. With the current data maybe a more general sentence like “the data support that inhibition of caspase-1 activation by EseJ depends on caspase-8 and NLRP3” would be better.

Response: Very helpful suggestions. The description of the caspase-8 and NLRP3 knockdowns have been moved to the Results section (lines 250-252) in the new revision, and more general statement had been made. Please see lines 336-340 in the revision “To further clarification, the caspase-8 and NLRP3 were knocked down in J774A.1 macrophages, and caspase-1 activation induced by the Δ *eseJ* strain was significantly diminished by either caspase-8 or NLRP3 knockdown (Fig. 3h & 3i). Collectively, the data support that EseJ-mediated suppression of caspase-1 activation depends on caspase-8 and NLRP3”.

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Edwardsiella piscicida type III protein EseJ suppresses apoptosis through down regulating type 1 fimbriae, which stimulate the cleavage of caspase-8, Tian Tian He, Ying Zhou, Ying Li Liu, Duan You Li, Pin Nie, Ai Hua Li, Hai Xia Xie

This paper is extension with few more additions. However, why the previous paper is not cited in this manuscript ?

Response: This is a misconception. That paper has been quoted several times in the manuscript, please see lines 70, 121, 168, 325, 340, and 422 in the original submission. That paper primarily reported that EseJ suppresses caspase-8-mediated apoptosis by down-regulating type 1 fimbriae (He et al., 2020). In the current work, it was found that EseJ suppresses cell apoptosis in murine macrophages at an early stage after infection, followed by a suppression of pyroptotic cell death at a late time point. Furthermore, T3SS-translocated EseJ inhibits PANoptosis by activating TAK1. So there is a misunderstanding here. And this study is definitely not an addition to the previous publication, but goes further in exploring the new discovery.

Major Comments

1. *Figure 9, the schematic is wrongly represented. All the arrows for apoptosis , pyroptosis etc are going up instead of going down. If the Authors are stating that EseJ inhibits Panoptosis, then all the form of death, arrows should go down in the presence of EseJ. In the absence of EseJ, the Panoptosis will go high.*

Response: The fork at EseJ indicates that EseJ is depleted and therefore PANoptosis levels are increased. In the revision we have made the labeling easier to see in the schematic.

2. *In vivo* fish model (fig 8) showed a marginal difference in their survival index showing that the WT bacteria-infected fish showed the least survival but the mutants also showed reduced survival.

Response: At the early post-infection stage (day 3), the mortality rates in WT-infected fish are significantly higher than that of Δ eseJ-infected fish, suggesting that EseJ contributes to the virulence of *E. piscicida*. The Δ eseJ strain fails to replicate inside the host cell and induces a significant increase in PANoptosis, which could lead to rapid activation of the immune system, forcing clearance of the Δ eseJ strain. Instead, the WT strain could disrupt host immune signaling, evade host clearance, replicate and spread in the host, leading to mass mortality. Mortality rates between WT- and Δ eseJ-infected fish are similar in the late post-infection period. This may be due to the fact that T3SS plays a major role in the early phase of infection, whereas T6SS plays a major role in the late phase (Yin et al., 2018; Li et al., 2021).

References:

Yin K, Guan Y, Ma R, Wei L, Liu B, Liu X, Zhou X, Ma Y, Zhang Y, Waldor MK, Wang Q. 2018. Critical role for a promoter discriminator in RpoS control of virulence in *Edwardsiella piscicida*. PLoS Pathog 14(8):e1007272.

Li DY, Liu YL, Liao XJ, He TT, Sun SS, Nie P, Xie HX. 2021. Identification and characterization of EvpQ, a novel T6SS effector encoded on a mobile genetic element in *Edwardsiella piscicida*. Front Microbiol 12:643498.

3. *All the caspase blots must have total caspase staining. Actin is fine, but total caspase must be stained with antibody to total caspase 8 or caspase 3, as the case may be.*

Response: This has been solved in the revision, please see new Fig. 3a, new Fig. 3d and new Fig. 3e in our revision.

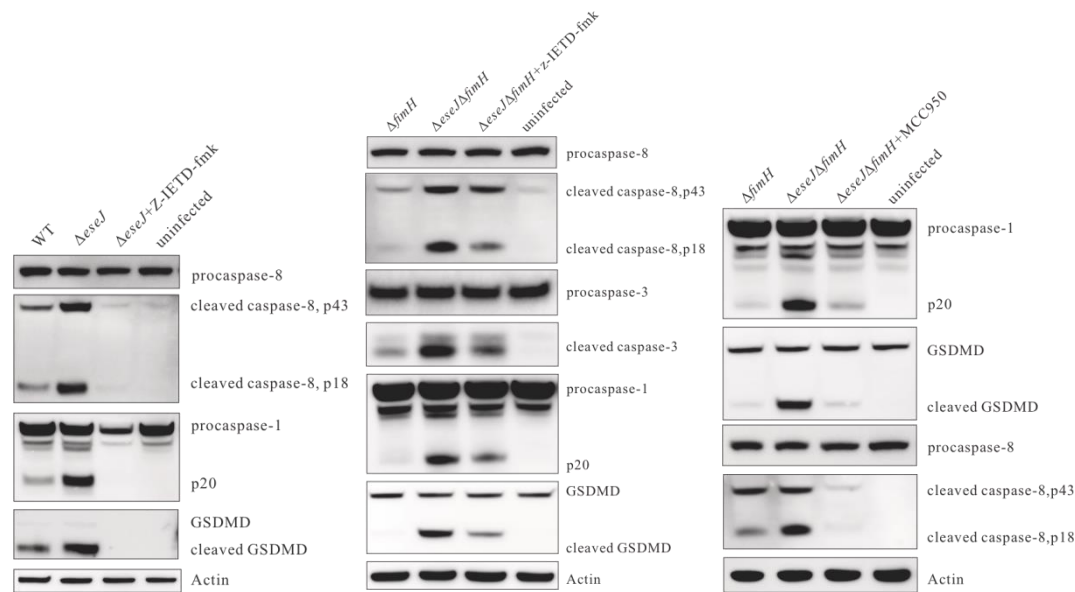


Figure legends:

Left panel: Immunoblotting of procaspase-8, cleaved caspase-8, cleaved caspase-1 and cleaved GSDMD from J774A.1 cells infected with *E. piscicida* WT or *ΔeseJ* strain at 3 hpi. J774A.1 cells were pretreated with the caspase-8 inhibitor Z-IETD-fmk for 1 h prior to infection with the *ΔeseJ* strain. Actin was used to confirm that similar amounts of protein were loaded per lane (new Fig. 3a).

Middle panel: Immunoblotting of procaspase-8, cleaved caspase-8, pro-caspase-3, cleaved caspase-3, cleaved caspase-1 and cleaved GSDMD from J774A.1 cells infected with *E. piscicida* *ΔfimH* or *ΔeseJΔfimH* strain at 3 hpi. J774A.1 cells were pretreated with the caspase-8 inhibitor Z-IETD-fmk for 1 h prior to infection with the *ΔeseJΔfimH* strain. Actin was used to confirm that similar amounts of protein were loaded per lane (new Fig. 3d).

Right panel: Immunoblotting of procaspase-8, cleaved caspase-8, cleaved caspase-1 and cleaved GSDMD from J774A.1 cells infected with *E. piscicida* *ΔfimH* or *ΔeseJΔfimH* strain at 3 hpi. J774A.1 cells were pre-treated with the NLRP3 inhibitor MCC950 for 1 h prior to infection with the *ΔeseJΔfimH* strain. Actin was used to confirm that similar amounts of protein were loaded per lane (new Fig. 3e).

4. Cytokine ELISA just be done for the gasdermin activation

Response: Very good suggestion. The release of the cytokines IL-1 β or IL-18 often follows the activation of caspase-1 and GSDMD. IL-1 β secretion levels were examined in J774A.1 cells infected with *E. piscicida* WT, *ΔeseJ*, and *ΔeseJ[eseJ]*

strains or *E. piscicida* $\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$, and $\Delta eseJ\Delta fimH$ strains. Please refer to the new Fig. 4o & 4p in our revision. Infection with the $\Delta eseJ$ strain stimulates a significant decrease in IL-1 β secretion. This is inconsistent with the observation that EseJ inhibits the activation of GSDMD and caspase-1. It is speculated that EseJ may play a role in the transcription or processing of pro-IL-1 β . This requires further study.

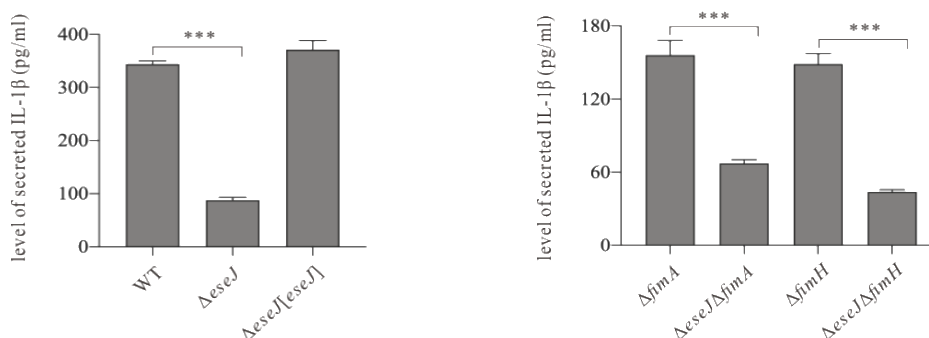


Figure legend: J774A.1 cells were infected with WT, $\Delta eseJ$, and $\Delta eseJ[eseJ]$ strains (Fig. 4o) or $\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$, and $\Delta eseJ\Delta fimH$ strains (Fig. 4p) for 5 h, after which culture supernatants were collected and the levels of IL-1 β secreted were assayed by ELISA. Supernatant from a non-infected cell culture was used as a control. Means $\pm$ SD of a representative experiment are shown. ***, $P < .001$.

5. With all the combinations of mutants and blocking caspases in macrophages, the bacterial burden must be measured. The CFU in macrophages under all different conditions and mutant constructs, with and without caspase inhibitors must be measured.

Response: Very good suggestion. The bacterial load in macrophages was examined at 3 h post infection in each experiment. The detailed results are outlined below:

Revised Fig. 2a-2g, 4a, 4b, 4i, and 4j: WT, $\Delta eseJ$, and $\Delta eseJ$ complemented with *eseJ* (via high-copy plasmid pACYC184 or low-copy plasmid pWSK29) strains (**Fig. a**);

Revised Fig. 2h-2k: WT, $\Delta eseJ$, $\Delta esaN$, and $\Delta eseJ\Delta esaN$ strains (**Fig. b**);

Revised Fig. 2l, 2m, 4c, 4d, 4k, and 4l: $\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains (**Fig. c**);

Revised Fig. 2n & 2o: WT, $\Delta eseJ$, $\Delta fimH$, and $\Delta fimH[eseJ]$ strains (**Fig. d**);

Revised Fig. 3a-3c: WT, $\Delta eseJ$, and $\Delta eseJ$ +Z-IETD-fmk (caspase-8 inhibitor) (**Fig. e**);

Revised Fig. 3d: $\Delta fimH$, $\Delta eseJ\Delta fimH$, and $\Delta eseJ\Delta fimH$ +Z-IETD-fmk (caspase-8 inhibitor) (**Fig. f**);

Revised Fig. 4e - 4g: $\Delta fimH$, $\Delta eseJ\Delta fimH$, $\Delta eseJ\Delta fimH$ +MCC950 (NLRP3 inhibitor) (**Fig. g**);

Revised Fig. 4m & 4n: WT, $\Delta eseJ$, WT+MCC950, and $\Delta eseJ$ +MCC950 (NLRP3 inhibitor)(**Fig. h**).

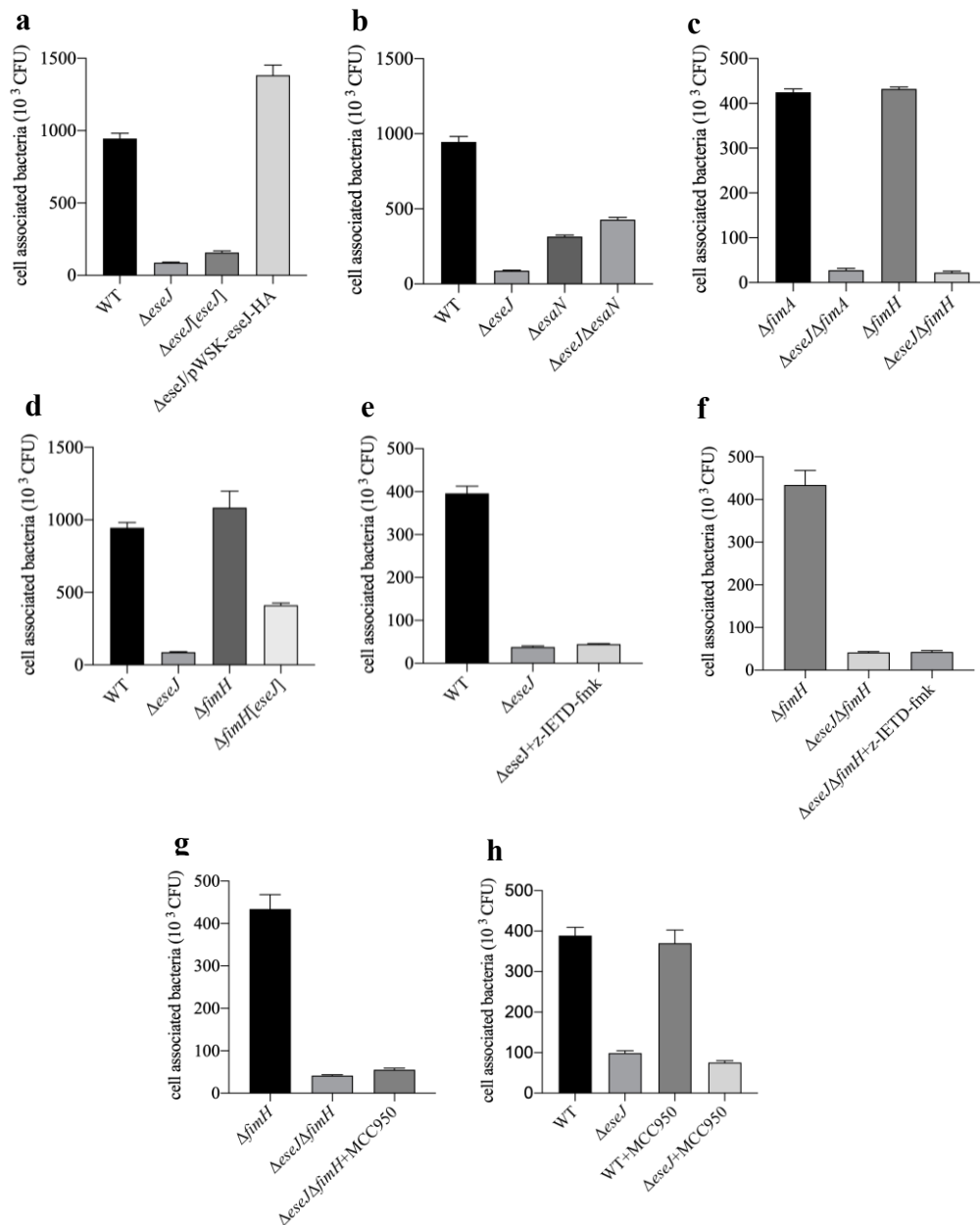


Figure legend: Quantification of cell-associated bacteria in J774A.1 cells infected with *E. piscicida* WT, $\Delta eseJ$, $\Delta eseJ[eseJ]$ and $\Delta eseJ/pWSK-eseJ-HA$ strains (**a**); or *E. piscicida* WT, $\Delta eseJ$, $\Delta esaN$ and $\Delta eseJ\Delta esaN$ strains at MOI of 10 (**b**); or *E. piscicida*

$\Delta fimA$, $\Delta eseJ\Delta fimA$, $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains at MOI of 20 (c); or *E. piscicida* WT, $\Delta eseJ$, $\Delta fimH$ and $\Delta fimH[eseJ]$ strains (d); or J774A.1 cells were pre-treated with caspase-8 inhibitor Z-IETD-fmk for 1 h before infection with the $\Delta eseJ$ strain (e); or J774A.1 cells were pre-treated with caspase-8 inhibitor Z-IETD-fmk for 1 h before infection with the $\Delta eseJ\Delta fimH$ strain (f); or J774A.1 cells were pre-treated with NLRP3 inhibitor MCC950 for 1 h before infection with $\Delta eseJ\Delta fimH$ strain (g); or J774A.1 cells were pre-treated with NLRP3 inhibitor MCC950 for 1 h before infection with WT, and $\Delta eseJ$ strains (h).

Reviewer #2 (Remarks to the Author):

In this manuscript, Tian Tian He and colleagues detail how the EseJ type III secretion effector from Edwardsiella piscicida inhibits PANoptosis. The data is convincing and thorough and should be of interest at least for all those studying host-pathogen interactions, in particular the role of effector proteins, and also for those interested in animal cell death pathways. The main weakness I notice is that almost all experiments are done in mice cells, while E. piscicida is a fish pathogen.

Do the same EseJ mechanism of inhibition of PANoptosis applies in fishes?

Response: As suggested, fish leukocytes were isolated from head kidney of mandarin fish and infected with $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains to examine LDH release (see new **Fig. 3h** in revision). A significant increase in cytolytic cell death in leukocytes, one of the hallmarks of pyroptosis or PANoptosis, was found to be stimulated by further deletion of EseJ in the $\Delta fimH$ strain. Consistent with the observation in murine macrophages, pretreatment fish leukocytes with the inhibitor of caspase-8 and NLRP3 before infection with the $\Delta eseJ\Delta fimH$ strain decreased the release of LDH. The $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains were chosen for infection because of their similar levels of adhesion and invasion.

Major issues:

1. E. piscicida is a fish pathogen and all experiments are done with a mouse macrophage cell line. I suppose that few reagents would be available to analyse mechanistic details in fish cell lines. But at least cell death/PI staining? Are the inhibitors used specific to mammals ?

Response: As suggested, the fish leukocytes from head kidney were isolated and

pre-treated prior to infection with *E. piscicida* strains using the caspase-8 inhibitor Z-IETD-fmk or the NLRP3 inhibitor MCC950. As shown in new **Fig. 3h**, deletion of *EseJ* stimulated a significant increase in LDH release. However, pretreatment with Z-IETD-fmk or MCC950 prior to infection dramatically reduced LDH release. The results indicate that through the caspase-8 and NLRP3 signaling pathways, *EseJ* inhibits lytic cell death in fish leukocytes. This is similar to the observation in murine macrophages.

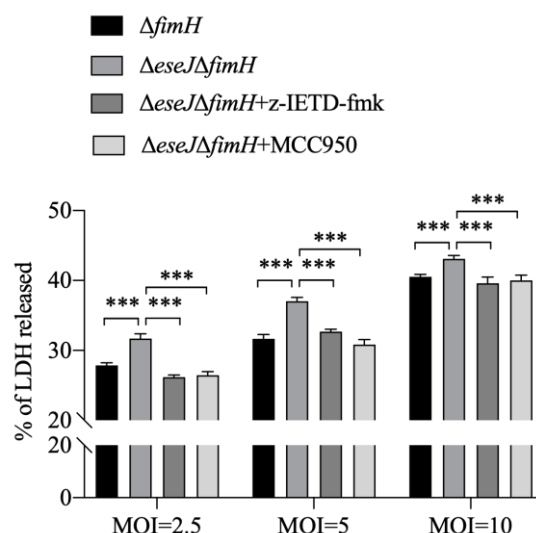


Figure legend: Fish leukocytes were treated with the caspase-8 inhibitor z-IETD-fmk or the NLRP3 inhibitor MCC950 for 1 h prior to infection with the $\Delta eseJ\Delta fimH$ strain at an MOI of 2.5, 5 or 10. Leukocytes infected with $\Delta fimH$ and $\Delta eseJ\Delta fimH$ strains were used as negative and positive controls, respectively. At 3 h post infection, culture supernatants were collected and the amount of LDH released was measured. ***, $P < .001$.

2. Figure 4e/f, there are many possible explanations (e.g., improper protein folding) that could explain why some truncated versions of *EseJ* function better than others. I suggest removing this data from the manuscript, as it also does not add much to the story.

Response: We agree. These two figures have been omitted in the revision.

3. Figure 6i, I suppose this was done at least twice. Showing the quantification of the immunoprecipitated caspase-8 and RIPK1 could support the authors conclusions.

Response: Very good suggestion. Quantification of immunoprecipitated caspase-8 and RIPK1 from three independent experiments is shown in new **Fig. 4m & 4n**. After

MCC950 pre-treatment, a significant decrease in immunoprecipitated caspase-8 and RIPK1 was detected in J774A.1 cells infected with the WT strain compared to the Δ *eseJ* strain.

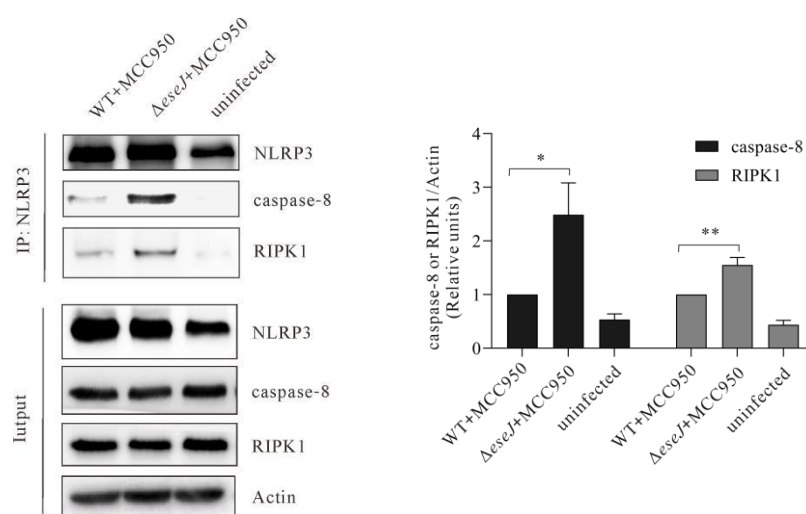


Figure legend

new Fig. 4m: Immunoblotting of the PANoptosome components RIPK1 and Caspase-8 in the input and in immunoprecipitation by the endogenous NLRP3 from lysates of J774A.1 cells which are pretreated with MCC950 prior to infection with WT and Δ *eseJ* strains.

new Fig. 4n: Quantitative analysis of caspase-8 and RIPK1 from immunoprecipitated samples shown in the left panel. The amount of caspase-8 or RIPK1 was quantified by densitometry and normalized to that of actin. The graph shows the relative ratios (Means $\pm$ SEM) of caspase-8 or RIPK1 from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

4. Most of the mechanistic data is based on the use of inhibitors. The authors should at least acknowledge this in their discussion as a limitation of the study. Key siRNA-mediated depletion or infection of cells from knock-out mice (MEFs) would make the conclusions stronger.

Response: Very helpful suggestion. We have acknowledged this limitation in the discussion, see lines 310 to 312. In fact, we have constructed plvx-*nlrp3*-siRNA and plvx-*caspase-8*-siRNA plasmids. These plasmids were packaged into lentiviruses and used to stably knock down NLRP3 and caspase-8 in J774A.1 cells. At 48 h post transduction, the cells were infected with Δ *fimH* or Δ *eseJ* Δ *fimH* strains at an MOI of

10. After 3 hours of infection, cells and supernatants were harvested and the expression levels of caspase-8, NLRP3, cleaved caspase-1 (p20) were examined by immunoblotting (Fig. S1). The *nlrp3* siRNA significantly reduced the protein level of NLRP3, indicating the knock down of NLRP3 in J774A.1 cells. Furthermore, transduction with *nlrp3* siRNA obviously reduced the level of cleaved caspase-1 (p20) in J774A.1 cells infected with the $\Delta\text{eseJ}\Delta\text{fimH}$ strain. Caspase-8 siRNA treatment also reduced caspase-1 levels. These results indicate that the activation of caspase-1 stimulated by infection with the $\Delta\text{eseJ}\Delta\text{fimH}$ strain is mediated by NLRP3 and caspase-8.

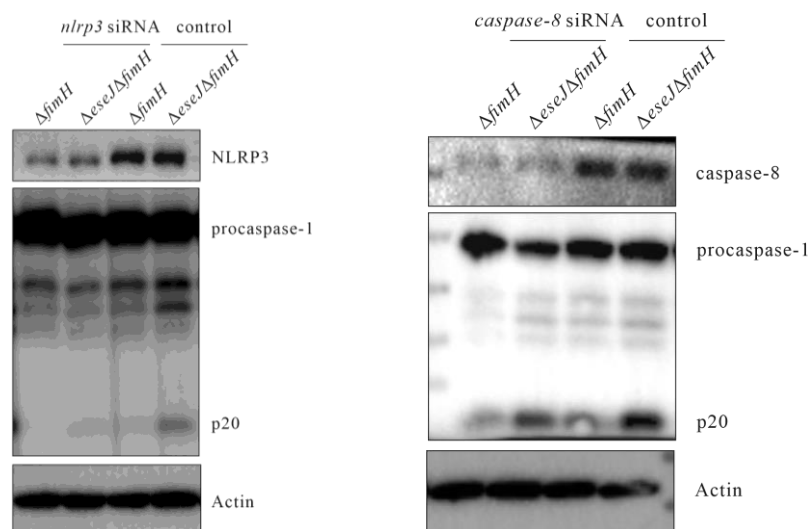


Figure legend: Immunoblotting of caspase-8, NLRP3, cleaved caspase-1 (p20) in J774A.1 cells transduced with plvx-*nlrp3*-siRNA (left panel) and plvx-*caspase-8*-siRNA (right panel) plasmids or plvx-control (non-sense sequence)-siRNA packaged into lentiviruses for 48 h prior to infection with ΔfimH or $\Delta\text{eseJ}\Delta\text{fimH}$ strains at an MOI of 10 for 3 h. The supernatants and cell lysates were harvested and probed with different antibodies. Actin was used to confirm that similar amounts of protein were loaded per lane.

Other/minor issues:

1. The title has a significant number of acronyms. I do not have an alternative but suggest it should be revised.

Response: It has been revised as “T3SS effector EseJ in *Edwardsiella piscicida* inhibits PANoptosis in macrophages”

2. *While the writing is generally OK, I also suggest it could be revised by someone with both English and scientific proficiency (e.g., "EseJ translocated" instead of "translocated EseJ").*

Response: Corrected.

3. *Line 21, Edwardsiella piscitida and not E. piscitida.*

Response: Corrected.

4. *Lines 54/55, a reference is missing for type III secretion system.*

Response: Corrected.

5. *Line 73, at least autophagy is also a form of PCD, and there may be others, less common. I suggest writing "...are three PCD pathways."*

Response: Corrected.

6. *Lines 81 and 82. Is this true for ALL pathogens?*

Response: Thank you for pointing this out. We have revised the sentence as "Many pathogens can stimulate one or more of these three PCD pathways during infection."

7. *Line 108, mechanisms is better than strategies (implying rational thinking, which bacteria do not have).*

Response: Corrected.

8. *Line 133, proteins do not have phenotypes; please revise the writing.*

Response: Corrected.

9. *Lines 135-137, there is no reference to a figure. I also find it a bit difficult by looking at figures 1 a or 2b to appreciate the cell rounding, cell swelling, and cell rupture of cells infected by the eseJ mutant strain. Maybe zoomed images or arrows could help.*

Response: It is Fig. 2a, this information has been added in the revision. In the new Fig. 3b, we have added annotated zoomed panels with arrows that explicitly highlight the Δ eseJ-induced morphological changes in J774A.1 cells - including cell rounding

at 1 hpi and cell swelling and rupture at 3 hpi.

10. Line 143, it seems full complementation by providing *eseJ* in trans; why "partially rescued?". For LDH release it does not seem so clear, but PI staining, yes.

Response: Yes, it is fully complementary for the LDH release, and on closer inspection, it is also fully complementary for the PI staining. This sentence has been rephrased in the revision.

11. Line 190, "delivered into host cells" would be better than "translocated into hosts".

Response: Corrected.

12. Figure 6i, typo in caspase 8.

Response: Corrected.

13. In Fig. 7, perhaps it would be easier to grasp if the legends hows the protein that is inhibited, instead of the chemical inhibitor.

Response: It has been changed as you suggested in the revision. This change makes it easier for the reader to follow the story.

14. It would make more sense to me if the story would start with the role of *EseJ* on virulence of *E. piscicida*, and then analyse how it works.

Response: Very helpful suggestion. The story begins with the role of *EseJ* in the virulence of *E. piscicida* in the revision.

RE: COMMSBIO-24-0939 “T3SS effector EseJ in *Edwardsiella piscicida* inhibits PANoptosis in macrophages”

Reviewer #1 (Remarks to the Author):

The Authors have answered all the queries in their revised manuscript.

Reviewer #2 (Remarks to the Author):

Overall, the authors have adequately addressed my previous concerns.

However, I still have a few minor points that should be considered:

1. Line 37: The correct term here is virulence, which is a quantitative term (i.e., can be increased or decreased), while pathogenicity is a qualitative term.

Response: Corrected.

2. Lines 191–192: I find this sentence somewhat confusing and repetitive relative to the introduction. Consider rephrasing to improve clarity, perhaps starting with "In previous studies..." or otherwise revising the introduction to this section.

Response: It has been rephrased as “In our previous study, we demonstrated that EseJ inhibits host cell apoptosis by specifically suppressing the type 1 fimbria-dependent activation of caspase-8 and following cleavage of caspase-3 (lines 206-207).”

3. Lines 203–206: The relevant detail is still difficult to visualize; I had to zoom in to 400% on my monitor. It may help to show the zoomed-in image directly in the "Cells" panel and to clearly indicate the zoomed regions in the original panels (for the 3 h time point). Consider adding a supplementary figure showing a wider field of the cells.

Response: Very helpful suggestion. In the new Fig. 3b, we have added an annotated, zoomed-in image to the ‘Cells’ panel. This image uses arrows to highlight the Δ *eseJ*-induced morphological changes in J774A.1 cells, including cell rounding at 1 hpi and cell swelling and rupture at 3 hpi. Additionally, stitched panorama images

were used to demonstrate wider-field views of Fig. 3b; these are provided in the supplementary information.

4. Lines 310–318: The description of the caspase-8 and NLRP3 knockdowns should be moved to the Results section rather than appearing in the Discussion. If possible, improve the quality of the immunoblot. Additionally, the statement beginning “Strikingly... NLRP3 knockdown (Fig. S1)” should be toned down, as the current evidence does not fully justify such a strong conclusion. With the current data maybe a more general sentence like “the data support that inhibition of caspase-1 activation by EseJ depends on caspase-8 and NLRP3” would be better.

Response: Very helpful suggestions. The description of the caspase-8 and NLRP3 knockdowns have been moved to the Results section (lines 250-252) in the new revision, and more general statement had been made. Please see lines 336-340 in the revision “To further clarification, the caspase-8 and NLRP3 were knocked down in J774A.1 macrophages, and caspase-1 activation induced by the Δ *eseJ* strain was significantly diminished by either caspase-8 or NLRP3 knockdown (Fig. 3h & 3i). Collectively, the data support that EseJ-mediated suppression of caspase-1 activation depends on caspase-8 and NLRP3”.