Cytokine storms and pyroptosis are primarily responsible for the rapid death of mice infected with pseudorabies virus

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Article citation details
R. Soc. open sci. 8: 210296.
http://dx.doi.org/10.1098/rsos.210296

Review timeline
Original submission: 24 February 2021
1st revised submission: 19 July 2021
2nd revised submission: 6 August 2021
Final acceptance: 6 August 2021

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History
RSOS-210296.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
No

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
No

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
Yes
Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
This paper has some merit addressing an important issue about the role of the immune response in the pathogenesis of the ADV infection in the mouse model. However, it needs a profound revision on the language, more detail in the figures texts, and a complete description of the experimental design (groups and number of animals). Also I would recommend to discuss the evident limitations of the mouse model when extrapolating to economical species suffering of Pseudorabies. The statistical results also should be more explicit in the text and figures to strengthen the discussion. See Appendix A.

Decision letter (RSOS-210296.R0)

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on behalf of Prof Malcolm White (Subject Editor)
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Reviewer comments to Author:
Reviewer: 1
Comments to the Author(s)
This paper has some merit addressing an important issue about the role of the immune response in the pathogenesis of the ADV infection in the mouse model. However, it needs a profound revision on the language, more detail in the figure legends, and a complete description of the experimental design (groups and number of animals). Also I would recommend to discuss the evident limitations of the mouse model when extrapolating to economical species suffering of Pseudorabies. The statistical results also should be more explicit in the text and figures to strengthen the discussion.

Going through the experimental design, it is not clear how the groups of animals were conformed. A total of eighty mice were divided into two groups (probably 40 control and 40 experimental), however, 6 animals of each group were killed at 4 different time points, given a total of 24 mice per group, ¿what happened with the rest of the animals?
On figure 2 six time points were described (36 mice), however, no results on the control group were shown.
All figures need a self-explanatory text. Figure 2 misses IL-18 text.

Although a statistical analysis is claimed to be applied on the results, differences were not shown in the text or on the figures. Since the groups of animals were not accurately described, the analysis must be detailed.

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Author's Response to Decision Letter for (RSOS-210296.R0)

See Appendix B.

RSOS-210296.R1 (Revision)

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Yes

Is the language acceptable?
No

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)

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This paper is now suitable for publication. However, requires a further review of the language to make it more understandable. Please check up the comments made on the yellow marked words or phrases, as well as on the stoke out words in the reviewed PDF file and be sure to amend the errors.
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| Manuscript ID | RSOS-210296 |
| Article Type: | Research |
| Date Submitted by the Author: | 24-Feb-2021 |
| Complete List of Authors: | Liu, Shanshan; Tongren Polytechnic College; National and Local Engineering Research Centre for Separation and Purification Ethnic Chinese Veterinary Herbs  
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Huang, Xuefei; Tongren Polytechnic College  
Yuan, Rui; Tongren Polytechnic College  
Yu, Jiansheng; Tongren Polytechnic College; National and Local Engineering Research Centre for Separation and Purification Ethnic Chinese Veterinary Herbs |
| Subject: | biotechnology < BIOLOGY, health and disease and epidemiology < BIOLOGY |
| Keywords: | cytokine stroms, pyroptosis, pseudorabies virus |
| Subject Category: | Biochemistry, Cellular and Molecular Biology |
Author-supplied statements

Relevant information will appear here if provided.

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*Does your article include research that required ethical approval or permits?:*
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**Conflict of interest**

I/We declare we have no competing interests

*Statement (if applicable):*
CUST_STATE_CONFLICT :No data available.

**Authors’ contributions**

This paper has multiple authors and our individual contributions were as below

*Statement (if applicable):*
Shanshan Liu did the molecular lab work and drafted the manuscript; Wei Sun conceived this study and participated in the data analysis and artwork making; Rui Yuan did the animal experiment. Xuefei Huang and Jiansheng Yu gave a finally revised the manuscript.
Cytokine stroms and pyroptosis are primarily responsible for the rapid death of mice infected with pseudorabies virus

Shanshan Liu¹,², Wei Sun¹*, Xuefei Huang¹, Rui Yuan¹,² and Jiansheng Yu¹,²

¹College of Agriculture, Tongren Polytechnic College, Bijiang District, Tongren City, Guizhou, 554300, China
²National and Local Engineering Research Centre for Separation and Purification Ethnic Chinese Veterinary Herbs, Tongren City, Guizhou, 554300, China

Keywords: cytokine stroms; pyroptosis; pseudorabies virus

Abstract

Pseudorabies virus (PRV), the causative agent of Aujeszky’s disease (AD), is one of the most harmful pathogens to pig industry. PRV has the ability to infect and kill a variety of mammals. Nevertheless, the underlying pathogenesis related to PRV still unclear. This study aim to investigate the pathogenesis induced by PRV in a mouse model. The mice infected with PRV-HLJ strain developed severe clinical manifestations at 36 hour post infection (hpi), and mortality occurred within 48–72 hpi. Hematoxylin eosin staining and qRT-PCR methods were used to detect the pathological damage and expression of cytokines related to immune in brain tissue, respectively. The cytokine stroms caused by IFN-α, IFN-β, TNF-α, IL-1β, IL-6, and IL-18 was related to the histopathological changes induced by PRV. This pattern of cytokine secretion clearly depicts an image of a typical cytokine storm, characterized by dysregulated secretion of pro-inflammatory cytokines and imbalanced pro-inflammatory and anti-inflammatory responses. In addition, the pyroptosis pathway was also activated by PRV through elevating the expression levels of nod-like receptor protein 3, caspase-1, gasdeimin-D and interleukin-1β/18. These findings provide a way for further understanding of the molecular basis in PRV pathogenesis.

1. Introduction

Pseudorabies virus (PRV) or suid α herpesvirus 1 is the pathogen of porcine Aujeszky disease (AD), which causes symptoms of respiratory system, nervous system and reproductive system¹. Besides pigs, many mammals are susceptible to PRV infection, such as cattle, sheep, rabbits, cats, dogs, guinea pigs, rats, and mice². However, pigs are the only susceptible animals that can survive, although the prognosis of the disease is critically dependent on the factors including inoculation site, virus strain and titer as well as age of pigs et. al³. PRV is known to cause severe encephalitis in piglets. Transmission mainly occurs through direct contact with oral and nasal secretions, but can also occur through aerosol through placenta or sexual intercourse¹. Therefore, the prevalence of PRV has led to a wide range of economic losses in the pork production industry. Inactivated and attenuated vaccines have been developed to delay or reduce swine death, but they are not yet able to eradicate the disease because none of them prevent potential infection and reactivation and shedding of virulence field virus⁴. Due to the serious impact of AD on pig industry, some countries are trying to eradicate AD basing on the DIVA(Differentiating Infected From Vaccinated animals) program. However, since 2011, the outbreak of AD occurred in farm pigs vaccinated with PR vaccine in China, which indicates that AD vaccine can not provide effective treatment to prevent wild PR infection⁵.

Mice and rabbits are usually used to study PRV in the laboratory. After infection, the animals showed abnormal excitement and nasal itching, accompanied by convulsions, and rapid death. In mice, PRV almost completely manifested as a neurogenic infection of the central nervous system(CNS), accompanied by fulminating central nervous symptoms and high mortality⁶. PRV is known to cause severe encephalitis in piglets, various non-native hosts, even in humans⁷. Few studies have focused on the pathogenesis of encephalitis. It known that pyroptosis involved in the immune response in various types of cell, which can be triggered by a variety of pathological stimuli, leading to the secretion of proinflammatory cytokines and intracellular contents⁸. Inflammation is a double-edged sword, which has a crucial role in metabolism. Mild inflammatory response could protect the body to a certain degree.

*Author for correspondence (sunwei_223@163.com).
†Present address: College of Agriculture, Tongren Polytechnic College, Bijiang District, Tongren City, Guizhou, 554300, China
help to repair damaged tissue, and be beneficial to steady-state reconstruction. Nevertheless, excessive inflammation may form a "cytokine storm", leading to tissue damage. In the present work, we describe the influence of PRV on the immune factor and pyroptosis-related factor in mice brain.

2. Materials and Methods

2.1 Reagents and animals

Annexin V-FITC/PI Apoptosis Kit was obtained from B D Company (Franklin Lakes, USA). Modified Bradford Protein Assay Kit, Antibody against NLRP3, Animal Total RNA Isolation Kit and Tissue Total Protein Extraction Kit were supplied by Sagon Biotech Company (Shanghai, China). Another antibody against Gasdermin D (GSDMD) was bought from Thermo Fisher Company (USA). IL-1β, IL-18 and β-actin antibodies were obtained from Bioss Company (Beijing, China). Caspase-1 antibody was obtained from Boster Biological Technology com.Ltd (Wuhan, Hubei, China). PrimeScript RT reagent Kit was bought from TAKARA company (Dalian, China). PRV-HLJ strain (MK080279.1) isolated Heilong Jiang was proved by Professor Jingfei Wang from Harbin Veterinary Research Institute, CAAS. Eighty 6-week-old female Balb/C mice were obtained from Dossy Experimental Animal Corporation (Chengdu, China).

2.2 Experimental

One week later, the mice were divided randomly into two groups, i.e control and pot-infection groups. The mice in the first group were injected via hypodermic injection with 0.2 mL of physiological normal saline (NS). The mice in the second group was received with 0.2 mL PRV-HLJ strain (10^4 TCID50). The animals were fed in the room illuminated with a 12 h light-dark cycle. The ambient temperature and relative humidity maintained at 22-24℃ and 40-60%, respectively. Ten mice were fed in each cage and given the above dosage. Water and diet was provided ad libitum. Six mice from each group were killed at 36, 48, 60 and 72 hour post infection (hpi), and their brain tissues were collected aseptically. Subsection specimens were snap frozen and stored at -80℃ for RNA extraction. In addition, portions of the brain were fixed in 4% paraformaldehyde solution for histopathological examination.

2.3 Histopathological analysis

Histopathological observation was operated by using a standard laboratory procedure. The brain was removed from experimental animals and washed thoroughly in phosphate buffered saline (PBS, pH 7.4). Then, the tissue was fixed in 4% paraformaldehyde for 2 d and transferred to 95% percent or absolute alcohol for dehydration. Thereafter, processed to paraffin embedding routine. The paraffin-embedded tissue was sliced into a 5 μm section, dewaxed in xylene and then rehydrated in graded alcohols. Section was stained with hematoxylin and eosin (H.E) staining and then examined under light microscope for histopathological examination.

2.4 Detection of pyroptosis by flow cytometry in brain

The rate of pyroptosis cells in brain was measured using an annexin V-FITC/PI Apoptosis kit according to the manuscript’s protocol. Brains were taken from mice, which were humanely killed at time above mentioned, ground to form suspension and filtered with a 300-mesh nylon screen. The cells were washed three times with pre-cold PBS and adjusted at a concentration of 1×10^6 cells/mL. Furthermore, 100 μL cells were incubated with annexin V-FITC/PI staining at room temperature for 15 min in a culture tube in the dark atmosphere. Each tube was added with 300 μL of binding buffer and then detected with a FCM (Becton Dickinson, USA). CellQuest Pro software (Becton Dickinson, USA) was used to visualize the results.

2.5 RNA extraction and qRT-PCR analysis

Total RNA from brains were isolated by a “Animal Total RNA Isolation Kit” according to the kit directions. RNA integrity was detected by 2% agarose gel electrophoresis. A spectrophotometer was used to detect the RNA quantity and quality (NanoDrop-2000, Thermofisher Company, USA). Total RNA was converted into DNA for qRT-PCR by using the PrimeScript RT reagent Kit. The first strand of cDNA was amplified through SYBR staining on a LightCycler 96 apparatus (Roche, Germany). All primers used in this research were designed by Oligo. 7 software and synthesized by Sagon Biotech Ltd., (Shanghai, China). Detailed information about primers were available in Table S1. 2^-ΔΔCt method was used for the analysis of mRNA expression. β-actin, a housekeeping gene, was used as an internal control for values correction.

2.6 Western blotting

A “Tissue Total Protein Extraction Kit” was used for protein extracted from brain. Protein concentration of each specimen was measured by Bradford assay. The proteins were first separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by being transferred onto a polyvinylidenedifluoride (PVDF) membrane. Then, the PVDF membrane was blocked with TBST solution contained with 5% non-fat milk at 4°C.
room condition for 2 h, and then incubated at 4°C condition for 12 h with the corresponding primary antibodies diluted with a solution: NLRP3(1:1000), Caspase-1(1:800), GSDMD(1:900), IL-1β(1:500), IL-1β(1:500) and β-actin(1:2000). Then, the PVDF membrane was incubated at room temperature with HRP-labeled secondary antibody for 1 h and blots were measured by an ECL reagent. The β-actin was used as a protein loading control.

2.7 Statistical analysis

SPSS22.0 software was used to data analyses. The results were represented as means ± standards deviations(mean±SD). One-way analysis of variance (ANOVA) package in SPSS 22.0 was used to evaluate the statistical significances between PRV and control group. GraphPad Prism6.0 software was used to perform statistical artworks. In all statistical comparisons, p value was introduced as a judgment to the statistical difference.

3. Results

3.1 Clinical symptoms and histopathological analysis

At 36 hpi, the mice infected with PRV generally showed typical clinical signs, including depression, anorexia and neuropathic itch. Mortality occurred within the period of 48-72 hpi. However, none of the mice in control group exhibited clinical symptoms or died.

Compared with the control group, there was a significant difference in the microscopic lesions in the PRV-inoculated mice (Figure 1). Normal brain structure was found in the control and PRV-inoculated mice before 36 hpi (Figure 1A and B). At 48 hpi, hyperemia appeared in brain tissue follow by perivascular space widened, perivascular lymphocytes increased, as well as degeneration and necrosis occurred in some neurons (Figure 1C). Focal lymphocytic infiltration was found in the brain at 60-72 hpi (Figure D and E).

3.2 The mRNA expression levels about innate immune-related genes

Innate immunity recognizes invading pathogens by binding to pattern recognition receptors, leading to the expression of antiviral molecules. Interferons(IFN) is an antiviral molecule, which has a pivotal role in the clearance of invading pathogenic microorganisms. In this work, the transcriptional levels of IFN-α, IFN-β and IFN-γ were determined by qRT-PCR.

As shown in Figure 2, the expression levels of IFN-α and IFN-β in the brain of PRV infected mice were up-regulated at 24 hpi and peaked at 48 hpi (Figure 2A-B). Interestingly, IFN-γ expression in brain was down-regulated before 36 hpi and then up-regulated until 60 hpi (Figure 2C). In addition, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-18) and anti-inflammatory cytokines (IL-4, IL-10) were also measured in the brain, respectively. The relative mRNA levels of TNF-α and IL-6 in brain were remarkably up-regulated caused by PRV infection, and peaked at 60 hpi followed by down-regulated but maintain at a high levels until 72 hpi (Figure 2D and F). After PRV infection, the IL-1β expression in brain was up-regulated from 24 hpi and lasted for the whole experiment period. However, IL-1β expression was up-regulated from 36 hpi and had a same trend as IL-1β. The cytokine stromes caused by IFN-α, IFN-β, TNF-α, IL-1β, IL-6 and IL-18 was related to the histopathological changes induced by PRV (Figure 1). Remarkably, the mRNA expression levels of IL-4 and IL-10 was up-regulated before 48 hpi and rapid down-regulated and maintained a low level until 72 hpi (Figure 2H and I). This indicated that PRV inhibited the expression of IL-4 and IL-10 since 48 hpi.

3.3 Detection of pyrolic cells in brain

Pyropstosis in brain was measured by Annexin V/PI double staining through flow cytometry. According to Figure 3, a large number of of PI+ pyropotic cells in brain observed at 36, 48, 60 and 72 hpi, which increased remarkably in a time-dependent way (p<0.01) compared with the control group (Table S2). In addition, PRV significantly up-regulated in situ the protein expression of caspase-1 (Figure 4B-D), IL-1β (Figure 4F-H) and IL-18 (Figure 4J-L) in the brain tissue in a time-dependent manner.

3.4 Changes of protein expression levels related to pyroptosis in the brain

To further investigate the effect of PRV on pyroptosis in vivo, the proteins involved in pyroptosis signal pathway were detected by western blotting, including NLRP3, Caspase-1, GSDMD, IL-1β and IL-18. From the result in figure 5, NRRP3 decreased at 36 hpi followed by an increasing trendcy in a time-dependent manner. In addition, the Caspase-1 level was significantly up-regulated by PRV from 24 hpi and maintained at a high level until the end of this experiment by comparing with that in the control group. Furthermore, GSDMD, an important pyroptosis marker protein, was also measured in all groups. As shown in Figure 5A, the amount of GSDMD was higher in all PRV group compared to the control. In addition, the expression levels of IL-1β and IL-18, two cytokines related to pyroptosis, were determined by western blotting. The result demonstrated that PRV could elevated the protein levels of IL-1β and IL-18.

3.5 Relative mRNA expression of genes related to pyroptosis in the brain
4. Discussion

PRV is a kind of neurophilic α herpesvirus, which belong to the genus Varicellovirus, family Herpesviridae. Pigs are the only natural hosts of PRV for their survival to infection. However, mice and rats can be naturally infected with PRV and cause a fatal disease. In lab, after intranasal infection in adult mice, PRV enters peripheral nerve cells and spreads to the central nervous system. Previous studies on PRV mainly focused on the pathogenicity and the resulting host immune response. The aim of our research was to describe the kinetics of cytokine secretion in vivo and to clarify whether cytokine storm involved in the pathogenesis of PRV. In this work, we identified cytokine storm and pyroptosis as the main causes of rapid death in mice infected with PRV.

Innate immune system is the first line in defending against the invasion of pathogens, accompanied by the recruitment of immune cells and the initiation of inflammatory response. Inflammation is an important process to solve microbial infection and a complex process involving the regulation of cytokine production. Dysfunction of these mechanisms can induce cytokine storms and related multiple organ failure. Inflammatory response is usually caused by a variety of pro-inflammatory cytokines, such as TNF, IL-1 and IL-6. These cytokines are the kind of pleiotropic proteins, which involve in the regulation of cell death in inflammatory tissue, vascular endothelial cell permeability, attracting the blood cells to inflammatory tissue, and acute phase proteins production.

Cytokines play an important role in all items of immune response, coordinating the innate and adaptive immune response. Consequently, in most cases, cytokines play a protective role in resisting endogenous and exogenous noxious stimuli, such as tissue injury and microbial invasion. IFNs are recognized as the central factors of antiviral infection, which has a pivotal role in innate immune response. In addition, Cytokines and interleukin play an important role in the pathogenesis of antiviral and viral infection. However, excessive immune activation and excessive release of cytokines could be rather pernicious. For example, overexpression of IFN-α, IL-1, and IL-6 in immune system could lead to vascular leakage, systemic fatigue, cardiomyopathy, vascular leakage, and acute phase proteins production.

In addition, persistent excessive IFN-α/β may also be harmful to immune system. In the present study, we found that a strong cytokine storm was induced by PRV in mice brain from 36-72 hpi, including the elevated expression levels of Type I IFNs (IFN-α and IFN-β) and Type II IFNs (IFN-γ) as well as proinflammatory factors. This storm was consistent with the histopathological changes in mice brain. Studies have shown that PRV could regulate the expression of cytokines, including type I and type II interferon and inflammatory factors, to establish a successful infection. Furthermore, IFN-α and IFN-β mediates a positive feedback regulation by binding to IFN-α and IFN-β receptor in an automatic or paracrine manner.

PRV infection causes apoptosis has been reported previously in vitro and in vivo. However, apoptosis is usually considered as an insoluble programmed cell death (PCD), which is characterized by an active programmed process of cell decomposition to avoid inflammation. The discrepancy found in this research may be induced by a new kind of PCD in cell death process. Pyroptosis is a new type of pro-inflammatory cell death, which is emerging as the mechanism of antagonizing and clearing pathogen infection, and requires the activation of caspase 1/4/5/11. In this research, the increasing trend of pyroptotic cells induced by PRV were detected in brain through flow cytometry. Furthermore, the expression of genes and proteins related to pyroptosis pathway were elevated by qRT-PCR and Western Blotting methods, respectively. Inflammmasomes are cytosolic sensors that could activate Caspase-1. Once activated, Caspase-1 has the ability to process and maturates IL-1β and IL-18 precursors, as well as cleave GSDMD, resulting in cell membrane channel opening and pyroptosis. Among the inflammmasomes, NLRP3 is currently the most well-known one, which responds to a variety of stimuli. NLRP3 was activated by PRV in this research. In addition, GSDMD, a key executor in pyrotosis, also be activated by PRV. This activity of GSDMD leads to the indirect release IL-1β and IL-18 from membrane pores. The pyroptotic cell-fate decision provides a large amount of inflammatory response at the site of infection. This was consist with the results from histopathological analysis (Figure 1) and immunohistochemistry (Figure 4), as well as a full explanation for cytokine storm caused by PRV in mice brain.

5. Conclusion

Cytokine storm and pyroptosis might be the main cause for the rapid death of mice inoculated with PRV strain. This results provided a new insight for further understanding the pathogenesis caused by PRV.
Data Accessibility

The datasets supporting this article have been uploaded in the form of “Supplementary Material”. In addition, the raw material related to this work are deposited at Dryad (https://datadryad.org/stash/dataset/doi:10.5061/dryad.zw3r2287j).

Competing Interests

The authors declare that there is no conflict of interests to this research.

Authors’ Contributions

Shanshan Liu did the qRT-PCR and Western blotting experiment and drafted the manuscript; Wei Sun conceived this study and participated in the data analysis and artwork making; Rui Yuan did the animal experiment. Xuefei Huang and Jiansheng Yu gave a finally revised the manuscript.

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Figure captions

Figure 1. Histopathological changes of brain of PRV-inoculated exam by H.E staining. (A) Microscopic lesion in the control group; (B-E) PRV-infected mice from 24, 48, 60 and 72 hpi.
Figure 2. The dynamic changes of mRNA expression related to immune induced by PRV. (A) IFN-α; (B) IFN-β; (C) IFN-γ; (D) TNF-α; (E) IL-1β; (F) IL-6; (G) IL-4; (H) IL-10

Figure 3. PRV caused pyroptosis in mice brain measured by flow cytometry. (A) Pre-infection; (B) 0 hpi; (C) 36 hpi; (D) 48 hpi; (E) 60 hpi; (F) 72 hpi,
**Figure 4.** In situ expression of caspase-1, IL-1β and IL-18 in mice brain. (A) Caspase-1 expression in the pre-infection group; (B-D) Caspase-1 expression in brain at 36, 60 and 72 hpi; (E) IL-1β expression in the pre-infection group; (F-H) IL-1β expression in brain at 36, 60 and 72 hpi; (I) IL-18 expression in the pre-infection group; (J-L) IL-18 expression in brain at 36, 60 and 72 hpi; Scale bar=50 μm.

**Figure 5.** The protein expression related to pyroptosis induced by PRV. (A) The relative amount of protein to β-actin; (B) protein expression related to pyroptosis measured by western blotting.
Figure 1. Histopathological changes of brain of PRV-inoculated examined by H.E staining. (A) Microscopic lesion in the control group; (B-E) PRV-infected mice from 24, 48, 60 and 72 hpi.
Figure 2. The dynamic changes of mRNA expression related to immune induced by PRV. (A) IFN-α; (B) IFN-β; (C) IFN-γ; (D) TNF-α; (E) IL-1β; (F) IL-6; (G) IL-4; (H) IL-10

250x181mm (300 x 300 DPI)
Figure 3. PRV caused pyroptosis in mice brain measured by flow cytometry. (A) Pre-infection; (B) 0 hpi; (C) 36 hpi; (D) 48 hpi; (E) 60 hpi; (F) 72 hpi; 99x65mm (300 x 300 DPI)
Figure 4. In situ expression of caspase-1, IL-1β and IL-18 in mice brain. (A) Caspase-1 expression in the pre-infection group; (B-D) Caspase-1 expression in brain at 36, 60 and 72 hpi; (E) IL-1β expression in the pre-infection group; (F-H) IL-1β expression in brain at 36, 60 and 72 hpi; (I) IL-18 expression in the pre-infection group; (J-L) IL-18 expression in brain at 36, 60 and 72 hpi; Scale bar=50 μm.
Figure 5. The protein expression related to pyroptosis induced by PRV. (A) The relative amount of protein to β-actin; (B) protein expression related to pyroptosis measured by western blotting.

150x61mm (300 x 300 DPI)
Dear editors and reviews,

Thank you for your kind comments on our manuscript entitled "Cytokine storms and pyroptosis are primarily responsible for the rapid death of mice infected with pseudorabies virus". Those comments are all valuable and very helpful for revising and improving our paper. We have studied the comments carefully and have revised the manuscript according to the reviews’ comments and suggestions. Revised portion are marked in red in the paper.

Responds to the editors:

All wrong spelling has been revised in the manuscript.

(1) Line 1 stroms

Response: we have revised the line 1, 20, 135, 169, 186, 206 and 219 storms.

(2) Line 21 respevtively.

Response: we have revised the line 20 : respectively.

(3) Line 32 lagrgedly

Response: we have revised the line 30 : largely.

(4) Line 38 virulence

Response: we have revised the line 36 : virulent.

(5) Line 39 basing

Response: we have revised the line 37 : based.

(6) Line 45 It known

Response: we have revised the line 37 : It is known.

(7) Line 48 proinflammatory

Response: we have revised the line 47 : pro-inflammatory.

(8) Line 52 pyroptosis-reliated

Response: we have revised the line 51: pyroptosis-related.

(9) Line 62 Eithty

Response: we have revised the line 11: Eighty.

(10) Line 75 photophate

Response: we have revised the line 75: phosphate.
(11) Line 87 visualized  
Response: we have revised the line 87: visualize.

(12) Line 95 were  
Response: we have revised the line 96: was.

(13) Line 97 correction.  
Response: we have revised the line 97: correction.

(14) Line 101 bradford  
Response: we have revised the line 101: Bradford.

(15) Lines 110-111: SPSS22.0 software was used to data analyses. The results were presented as means ± standards deviations (mean±SD)  
Response: we have revised the line 110-111: SPSS22.0 software was used for data analyses. The results were presented as means ± standards deviations (mean±SD).

(16) Line 150, 197 trendency  
Response: we have revised the line 153 and 203: tendency.

(17) Line 151 manitained  
Response: we have revised the line 154: maintained.

(18) Line 152 compareing and 161 comparint  
Response: we have revised the line 155: comparing it, and 162 compared.

(19) Line 154 levles  
Response: we have revised the line 157: levels.

(20) Line 164 belong  
Response: we have revised the line 169: belongs.

(21) Line 205 consist  
Response: we have revised the line 212: consistent.

(22) Line 226 wirk  
Response: we have revised the line 229: work.

Responds to the reviews’ comments:

Reviewer: 1

Comments to the Author(s)

This paper has some merit addressing an important issue about the role of the immune response in the pathogenesis of the ADV infection in the mouse model.

(1) However, it needs a profound revision on the language, more detail in the figure legends, and a complete description of the experimental design (groups and number of animals).

Response: we have revised thought the manuscript to enhance the expression, grammar constructions and word spelling.

Figure legends have been improved in figure 1-5 in lines: 387-420.
Eighty 6-week-old female Balb/C mice were used in this study. We have revised in lines 64-65: “One week later, the mice were divided randomly into five groups with sixteen per group, including one control group and four experimental groups, which mice were infected at 36 hpi, 48 hpi, 60 hpi and 72 hpi, respectively.”

(2) Also I would recommend to discuss the evident limitations of the mouse model when extrapolating to economical species suffering of Pseudorabies.

**Response:** We have revised in lines 216-218: “Although cytokine storms and pyroptosis might be the cause for the rapid death of mice caused by PRV strain. However, due to the difference of immune system between mice and pig, more detail information about the pathogenesis to pig and other mammal animals need to be further clarify in future.”

(3) The statistical results also should be more explicit in the text and figures to strengthen the discussion.

**Response:** We have revised in “3.2 The mRNA expression levels about innate immune-related genes” from line 125-142.

Figure 1-4 have been improved to strengthen the results such as marking the statistical difference, and clarifying the the results and discussion sections.

(4) Going through the experimental design, it is not clear how the groups of animals were conformed. A total of eighty mice were divided into two groups (probably 40 control and 40 experimental), however, 6 animals of each group were killed at 4 different time points, given a total of 24 mice per group, what happened with the rest of the animals?

**Response:** We have revised in lines 64-65 to clarify the animal groups in this work: “One week later, the mice were divided randomly into five groups with sixteen per group, including one control group and four experimental groups, which mice were infected at 36 hpi, 48 hpi, 60 hpi and 72 hpi, respectively.”

(5) On figure 2 six time points were described (36 mice), however, no results on the control group were shown. All figures need a self-explanatory text. Figure 2 misses IL-18 text.

**Response:** all statistical results were compared with that in the control group. And figure coordinate has been relabeled so as not to cause misreading.

All figure legend have been modified and IL-18 text in figure 2 has been added in line 398.

(6) Although a statistical analysis is claimed to be applied on the results, differences were not shown in the text or on the figures. Since the groups of animals were not accurately described, the analysis must be detailed.

**Response:** we have added the statistical difference in the results of manuscript and figure 2.

The animals used in this study has been clearly described in the line 64-65. And the detail information about the results and discussion has been revised in the manuscript.
The other sections revised have been marked in red in the manuscript.

Thanks again for the excellent and professional revision of our manuscript. Hopefully, we could have our article been considered of publication in this journal. Should there been any other corrections we could make, please feel free to contact us by email. My email is sunwei_223@163.com.

Yours sincerely,

Wei Sun

July 18th, 2021
Dear editors and reviews,

Thank you for your kind comments on our manuscript entitled “Cytokine storms and pyroptosis are primarily responsible for the rapid death of mice infected with pseudorabies virus”. Those comments are all valuable and very helpful for revising and improving our paper. We have studied the comments carefully and have revised the manuscript according to the reviews’ comments and suggestions. Revised portion are marked in red in the paper.

Responds to the reviews’ and editors’ comments:

Reviewer: 1
Comments to the Author(s)
This paper is now suitable for publication. However, requires a further review of the language to make it more understandable.

Response: With the help of INCRESCIENCE co., ltd (https://check.newacademic.net, a service resources purchased by our college), we have made extensive amendments on the manuscript to improve clarity, enhance expression and grammar constructions provide by the helping. We believe this revised manuscript is greatly improved in language use.

The detailed modification information is as follows:
unspecified location of the current answers (if any) were not provided. This is a challenging task for large language models, as they need to understand and answer questions based on the text content. The document contains scientific information, and understanding it requires knowledge in the respective fields. The text appears to be in Chinese, which may further complicate the task for non-native speakers. The model might need additional context or training to accurately answer questions based on this text. Some patients may also have unique experiences or knowledge that could help in understanding the text. It's important to note that the text content is not clearly visible, and the model might not be able to provide a precise response without further clarification or context.
leaves of 1055, 385, 280, 60, and 0 ng/ml of FGF were generated on the surface of the original FGF-seeded compound and then followed by treatment with a fibronectin-seeded compound. The data showed that the FGF-seeded compound could enhance the proliferation of fibroblasts and that the initial FGF concentration was inversely proportional to the FGF-seeded compound.

In conclusion, the results of this study suggest that FGF-seeded compounds can enhance the proliferation of fibroblasts and that the initial FGF concentration is inversely proportional to the FGF-seeded compound. Further studies are needed to investigate the potential clinical applications of these findings.
Please check up the comments made on the yellow marked words or phrases, as well as on the stoke out words in the reviewed PDF file and be sure to amend the errors.

(1) Line 17 PRV

**Response:** Pseudorabies virus can be abbreviated as PRV or PrV according the references.

(1) Line 22 immune

**Response:** we have revised in line 19 “ immune reaction”.

(2) Line 24 a

**Response:** we have deleted “a” line 21.

(3) Line 31 Besides

**Response:** we have deleted “a” line 28 “Many mammals, including pigs, ............”.

(4) Line 34 et al

**Response:** we have deleted “a” line 31 “........as well as the age of pigs”.

(5) Line 36 through

**Response:** we have revised in line 33 “and”

(6) Line 67 respectively

**Response:** we have deleted “respectively” line 65.

(7) Line 69...was...of...

**Response:** we have revised in line 67 “...were received...”

(8) Line 84 protocol

**Response:** we have revised in line 82 “...instructions provided”.

(9) Line 92 directions

**Response:** we have revised in line 90 “... instructions”.

(10) Line 117 showed

**Response:** we have revised in line 116 “...appeared...”.

(11) Line 131 up

**Response:** we have revised in line 130 “...down-regulated...”.

(12) Line 136 but maintain at a high level until 72 hpi (p<0.01 and p<0.05, Figure 2D and F)

**Response:** we have revised in line 135 “...by down-regulated at 72 hpi (Figure 2 D and F)”.

(13) Line 138 However
Response: we have revised in line 136-137 “In addition...”.

(14) Line 143 pyroplic

Response: we have revised in line 142 “...pyroptosis...”.

(15) Line 146 than

Response: we have revised in line 145 “...that...”.

(16) Line 152 Caspae-1

Response: we have revised in line 151 “Caspase-1”.

(17) Line 166 Varicelloviru

Response: we have revised in line 168 “Varicellovirus”.

(18) Lines 212-213 detail information about the

Response: we have revised in line 215-216 “detailed information about the pathogenesis to pig and other mammal animals need to be further clarified in the future”.

(19) Lines 152, 158 and 202 western

Response: we have deleted western in lines 151, 157 and 204.

The other sections revised have been marked in red in the manuscript.

Thanks again for the excellent and professional revision of our manuscript. Hopefully, we could have our article been considered of publication in this journal. Should there been any other corrections we could make, please feel free to contact us by email. My email is sunwei_223@163.com.

Yours sincerely,

Wei Sun