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

Wei Sun, Shanshan Liu, Xuefei Huang, Rui Yuan and Jiansheng Yu

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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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The Editors assigned to your paper RSOS-210296 "Cytokine stroms and pyroptosis are primarily responsible for the rapid death of mice infected with pseudorabies virus" have now received comments from reviewers and would like you to revise the paper in accordance with the reviewer comments and any comments from the Editors. Please note this decision does not guarantee eventual acceptance.

We invite you to respond to the comments supplied below and revise your manuscript. Below the referees' and Editors' comments (where applicable) we provide additional requirements. Final acceptance of your manuscript is dependent on these requirements being met. We provide guidance below to help you prepare your revision. There are particular concerns regarding the quality of the written English - please seek advice from a service such as https://royalsociety.org/journals/authors/benefits/language-editing/ before resubmitting.

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on behalf of Prof Malcolm White (Subject Editor)
openscience@royalsociety.org

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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-- An individual file of each figure (EPS or print-quality PDF preferred [either format should be produced directly from original creation package], or original software format).
-- An editable file of each table (.doc, .docx, .xls, .xlsx, or .csv).
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Note: you may upload the figure, table, and caption files in a single Zip folder.
-- Any electronic supplementary material (ESM).
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Author's Response to Decision Letter for (RSOS-210296.R0)

See Appendix B.

RSOS-210296.R1 (Revision)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

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?
No

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

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. 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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Author's Response to Decision Letter for (RSOS-210296.R1)

See Appendix C.

Decision letter (RSOS-210296.R2)

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on behalf of Malcolm White (Subject Editor)
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Appendix A

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

Journal:	*Royal Society Open Science*
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 Sun, Wei; Tongren Polytechnic College 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.

Ethics

Does your article include research that required ethical approval or permits?:
Yes

Statement (if applicable):
The authors declare that this manuscript is original, which has not been published before and is also not currently being considered for publication elsewhere.
All animal procedures were approved by the Tongren Polytechnic College Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Data

It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:
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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.

ROYAL SOCIETY OPEN SCIENCE

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

Shanshan Liu[1,2], Wei Sun[1]*, Xuefei Huang[1], Rui Yuan[1,2] and Jiansheng Yu[1,2]

[1]*College of Agriculture, Tongren Polytechnic College,Bijiang District, Tongren City, Guizhou, 554300, China*
[2] *National 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 indusry. 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 starin 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, respevtively. The cytokine stroms caused by IFN-α, IFN-β, TNF-α, IL-1β, IL-6 and IL-18 was related to the histopatholigical 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 system[1]. Besides pigs, many mammals are susceptible to PRV infection, such as cattle, sheep, rabbits, cats, dogs, guinea pigs, rats and mice[2]. However, pigs are the only susceptible animals that can survive, although the prognosis of the disease lagrgedly depends on the factors including inoculation site, virus strain and titer as well as age of pigs *et. al*[3]. AD is a highly infectious disease with high mortality in piglets. Transmission mainly occurs through direct contact with oral and nasal secretions, but can also occur through aerosol through placenta or sexual intercourse[1]. 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 virus[4]. 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 infection[5].

Mice and rabbits are usually used to study PRV in the laboratory. After infection, the animals showed show 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 fulminant central nervous symptoms and high mortality[6]. PRV is known to cause severe encephalitis in piglets, various non-native hosts, even in humans[7]. 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 contents[8]. Inflammation is a double-edged sword, which has a crucial role in metabolism. Mild inflammatory response could protect the body to a certain degree,

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help to repair damaged tissue, and be feneficial 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-reliated 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-1antibody was obtained from Boster Biological Technology com.Ltd (Wuhan, Hubei, China). PrimeScript RT reagent Kit was bought from TAKARA company (Daliang, China). PRV-HLJ strain (MK080279.1) isolated Heilong Jiang was provied by Professor Jingfei Wang from Harbin Veterinary Research Institute, CAAS. Eithty 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 $TCID_{50}$). 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 nap frozen and stored at -80˚C for RNA extraction. In addition, portions of the barin 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 aninals and washed thoroughly in photophate 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 visualized 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. $2^{-\Delta\Delta Ct}$ method was used for the analysis of mRNA expression. β-actin, a housekeeping gene, was used as an internal control for values corrcetion.

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) follwed by being transferred onto a polyvinylidedne difluoride (PVDF) membrane. Then, the PVDF membrane was blocked with TBST solution contained with 5% non-fat milk at

room condition for 2 h, and then incubated at 4℃ condition for 12 h with the corresponding primary antibodies diluted with a sulution: NLRP3(1:1000), Caspase-1(1:800), GSDMD(1:900), IL-1β(1:500), IL-1β(1:500) and β-action(1:2000). Then, the PVDF membrane was incubated at room temperature with HRP-labeled secondary antibody for 1 h and blots were measued by a 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 espresented 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 infliltration 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[9]. In this work, the transcriptional levels of IFN-α, IFN-β and IFN-γ were determinated 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). Interesting, 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 remarkedly 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 stroms caused by IFN-α, IFN-β,TNF-α, IL-1β, IL-6 and IL-18 was related to the histopatholigical changes induced by PRV(Figure 1). Remarkedly, the mRNA expression levels of IL-4 and IL-10 was up-reguated 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

Pyroptosis in brain was measured by Annexin V/PI double staining through flow cytometry. According to Figure 3, a large number of of PI+ pyroptotic cells in brain observed at 36, 48, 60 and 72 hpi, which increased remarkedly in a time-dependent way ($p<0.01$) compared with than in 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, Caspae-1, GSDMD, IL-1β and IL-18. From the result in figure 5, NRRP3 decresed at 36 hpi followed by an increasing tredency in a time-dependent manner. In addition, the Caspase-1 level was significantly up-regulated by PRV from 24 hpi and manitained at a high level until the end of this experiment by compareing 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 levlels of IL-1β and IL-18, two cytokines related to pyroptosis, were determinated by western blotting. The result demonstrated that PRV could elevated the protein levlels of IL-1β and IL-18.

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

The mRNA expression levels of pyroptosis-related factors were further detected by qRT-PCR in this research. As shown in Table S3, the mRNA expression level of *NRRP3* were markedly increased (p<0.05) in a time-dependent manner following by PRV treatment. Moreover, the mRNA expression levels of *Caspase-1* in the PRV inoculation groupswere also up-regulated (p<0.01) when by comparint that among groups. Besides, the *GSDMD* mRNA expression level was up-regulated in an increased trendency (p<0.01) compared with that in the pre-infection group.

4. Discussion

PRV is a kind of neurophilic α herpesvirus, which belong to the genus *Varicelloviru*, family *Herpesviridae*. Pigs are the only natural hosts of PRV for their survive 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[10]. 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 strom and pyroptosis as the main causes of rapid death in mice infected with PRV.

Innate immune system is the first line in defensing against the invasion of pathogens, accompanied by the recruitment of immune cells and the initiation of inflammatory response[11]. 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[12]. 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~~ involves 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[13].

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[14]. 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[15]. For example, overexpression of TNF-α, IL-1, and IL-6 in immune system could lead to vascular leakage, systemic fatigue, cardiomyopathy, ~~vascular leakage,~~ and acute phase protein synthesis[15]. In addition, persistent excessive IFN-α/β may also be harmful to immune system[14]. In the present study, we found that a strong cytokine strom was induced by PRV in mice brain from 36-72 hpi, including the elevated expression levles of Type I IFNs (IFN-α and IFN-β) and Type Ⅱ IFNs (IFN-γ) as well as proinflammatory factors. This strom was consitent 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 eatablish a successful infection[16, 17]. 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[18].

PRV infection causes apoptosis has been reproted previously *in vitro* and *in vivo*[19, 20]. 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[21]. 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[22]. In this research, the increasing tredency 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. Inflammasomes are cytosolic sensors that could activate Caspase-1[23]. 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[8]. Among the inflammasomes, NLRP3 is currently the most well-known one, which responds to a variety of stimuli. NLRP3 was accivated by PRV in this research. In addition, GSDMD, a key executor in pyrotosis[24], also be activated by PRV. This activity of GSDMD leads to the indirect release IL-1β and IL-18 from membrane pores[25]. 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 strom caused by PRV in mice brain.

5. Conclusion

Cytokine stroms 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.

Acknowledgments

Many thanks to Jiangtao Feng for his helping in animal administration.

Ethical Statement

This study was approved by Animal Care and Use Committee of Tongren Polytechnic College. All animal administrations, sample collection and procedures were performed by the guidelines approved.

Funding Statement

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Data Accessibility

The datasets supporting this article have been uploaded in the form of "Supplementary Material". In addition, the raw material related to this wirk are deposited at Dryad (https://datadryad.org/stash/dataset/doi:10.5061/dryad.zw3r2287j) [25].

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

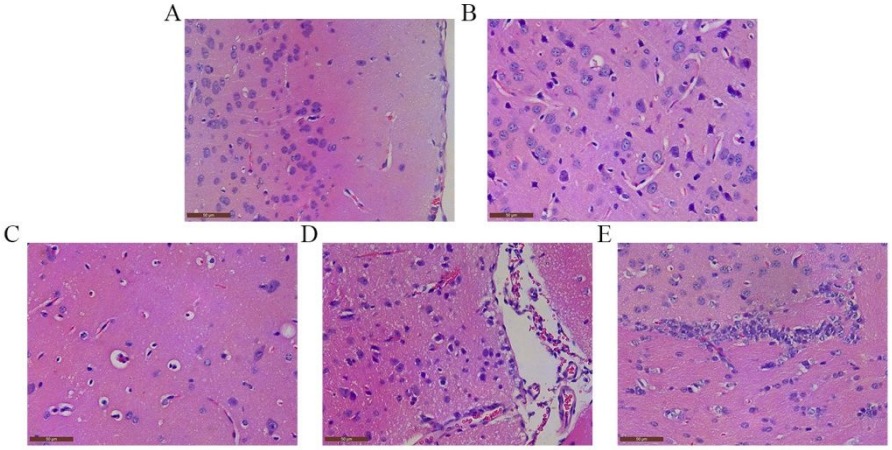

Figure 1. Histopatholigical changes of brain of PRV-inoculated examed by H.E staning. (A) Microscopic lesion in the control group; (B-E) PRV-infected mice from 24, 48, 60 and 72 hpi.

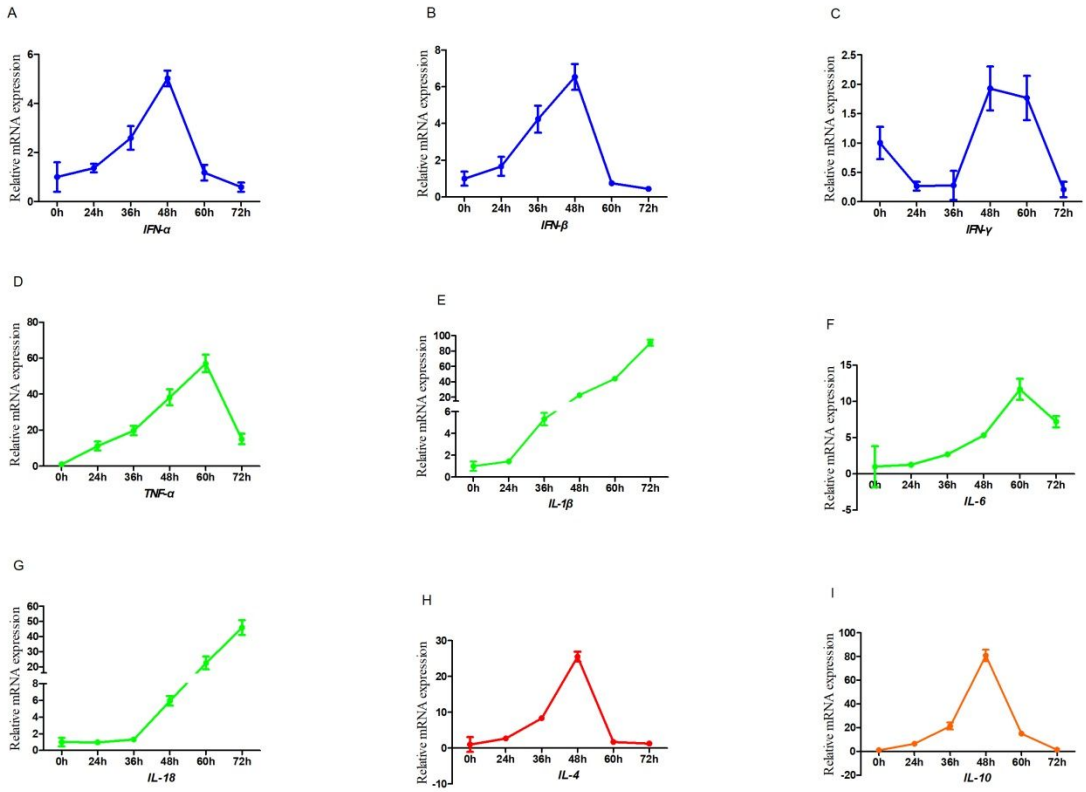

Figure 2. The dynamic changes of mRNA expression related to immune induced by PRV. (A) *IFN-α*; (B) *IFN-β*; (C) *IFN-γ*; (D) *TNF-α*; (E) *IL-1β*; (F) *IL-6*; (G) *IL-4*; (H) *IL-10*

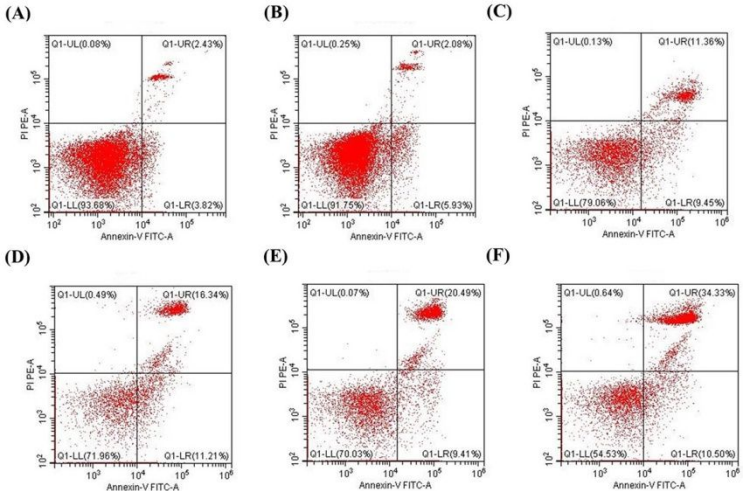

Figure 3. PRV caused pyroptosis in mice brain measured by flow cytometry. (A) Pre-infection; (B) 0 hpi; (C) 36 hpi; (D) 48 hpi; (E) 60 hpi; (F) 72 hpi;

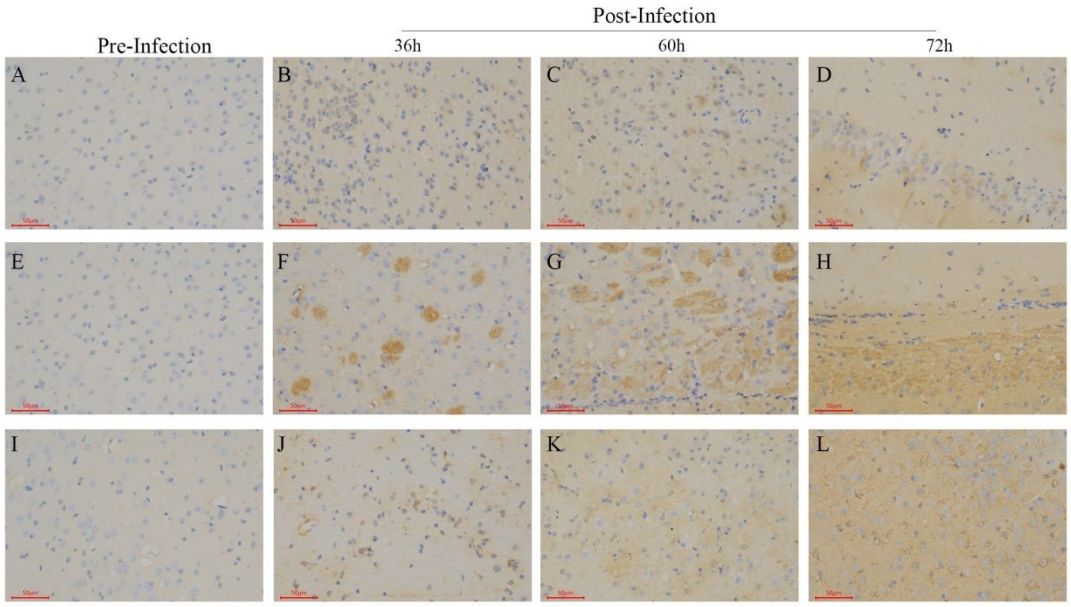

Figure 4. In situ expression of caspase-1, IL-1β and IL-18 in mice brain. (A) Caspase-1 expression in the pre-infection group; (B-D) Caspase-1 expression in brain at 36, 60 and 72 hpi; (E) IL-1β expression in the pre-infection group; (F-H) IL-1β expression in brain at 36, 60 and 72 hpi; (I) IL-18 expression in the pre-infection group; (J-L) IL-18 expression in brain at 36, 60 and 72 hpi; Scale bar=50 μm.

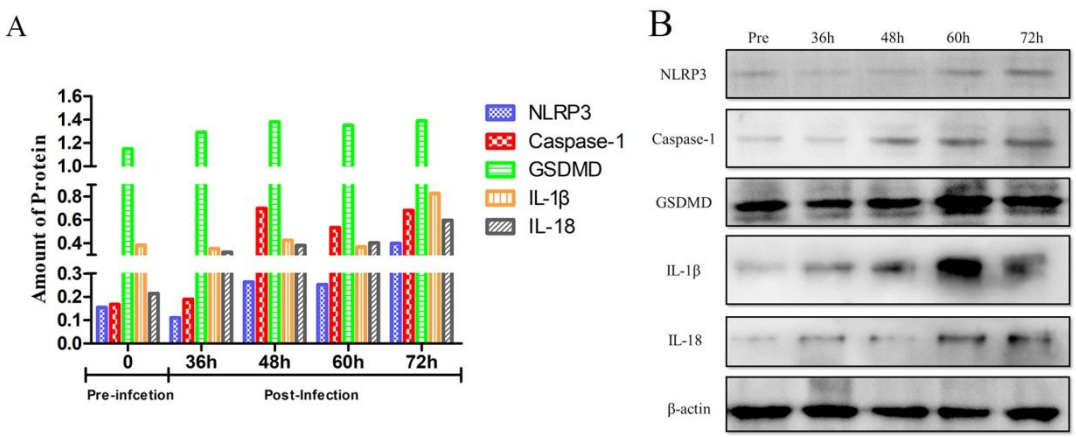

Figure 5. The protein expression related to pyroptosis induced by PRV. (A) The relative amount of protein to β-actin; (B) protein expression related to pyroptosis measued by western blotting.

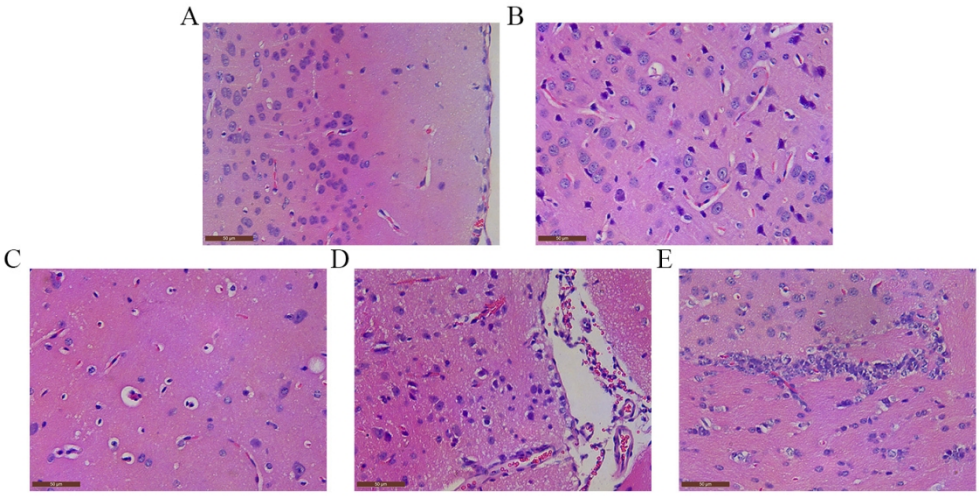

Figure 1. Histopatholigical changes of brain of PRV-inoculated examed by H.E staning. (A) Microscopic lesion in the control group; (B-E) PRV-infected mice from 24, 48, 60 and 72 hpi.

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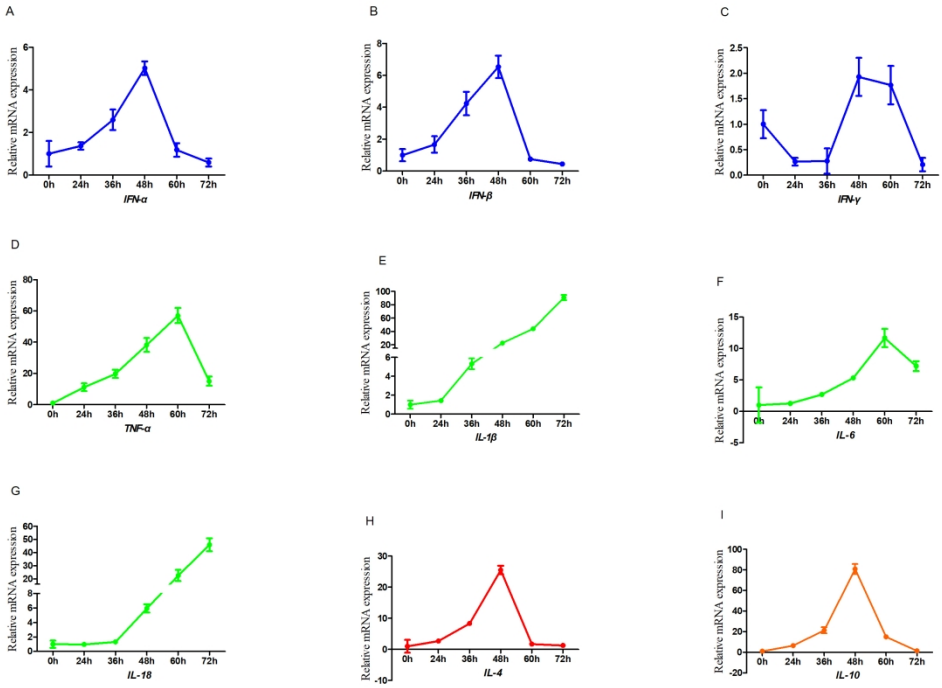

Figure 2. The dynamic changes of mRNA expression related to immune induced by PRV. (A) IFN-α; (B) IFN-β; (C) IFN-γ; (D) TNF-α; (E) IL-1β; (F) IL-6; (G) IL-4; (H) IL-10

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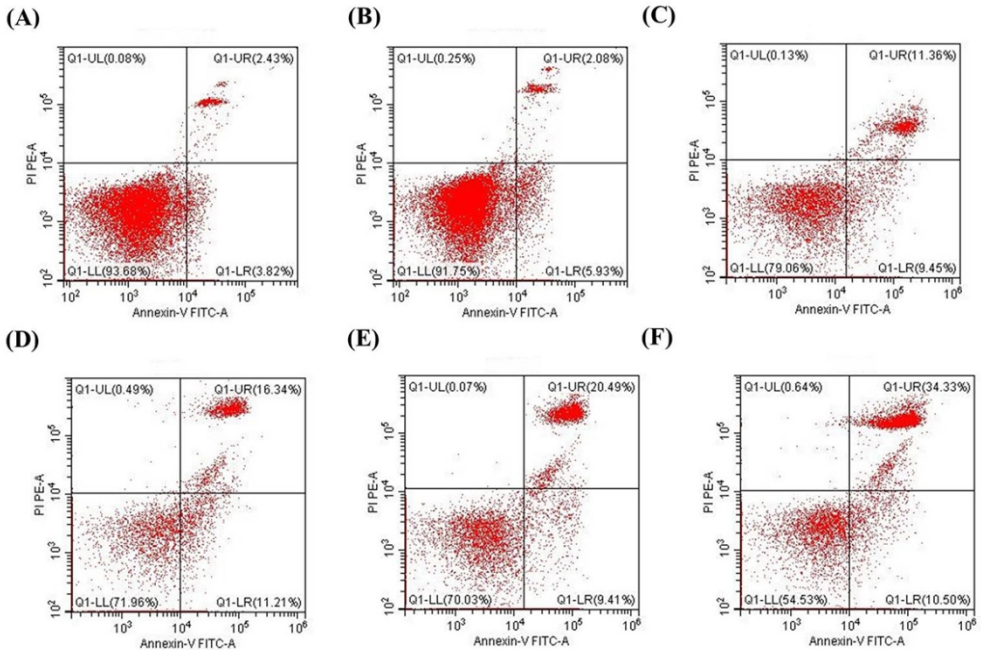

Figure 3. PRV caused pyroptosis in mice brain measured by flow cytometry. (A) Pre-infection; (B) 0 hpi; (C) 36 hpi; (D) 48 hpi; (E) 60 hpi; (F) 72 hpi;

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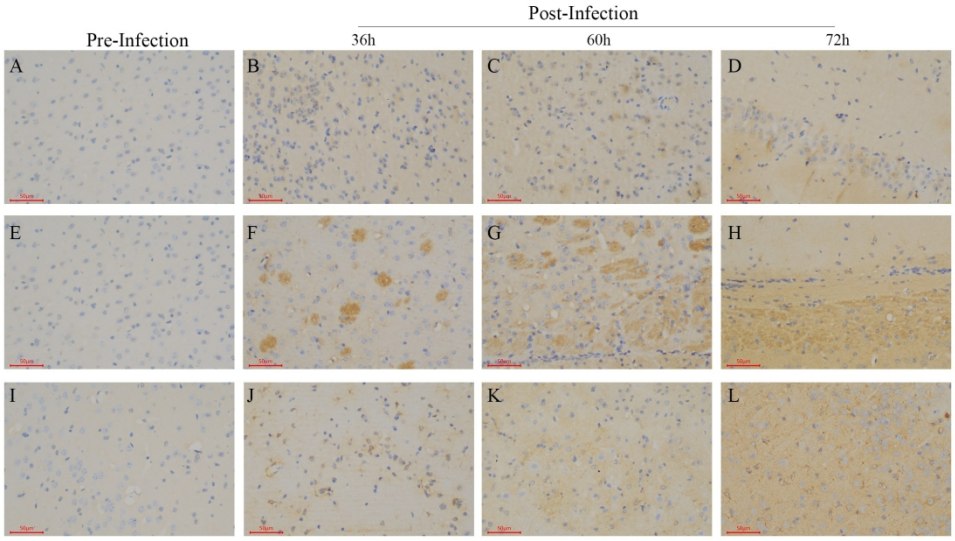

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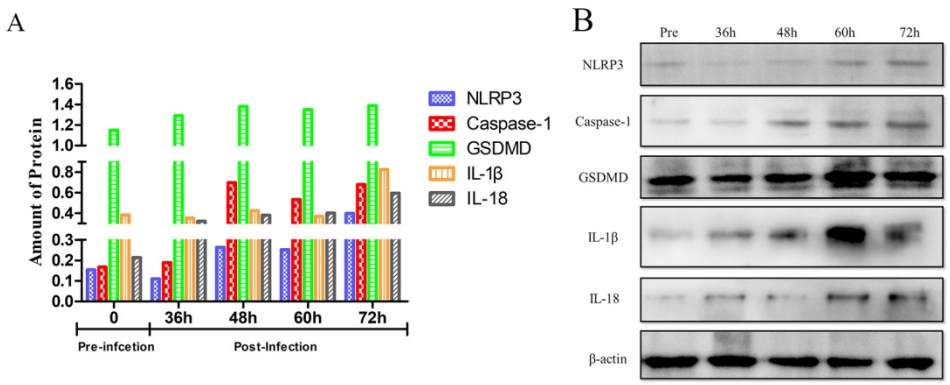

Figure 5. The protein expression related to pyroptosis induced by PRV. (A) The relative amount of protein to β-actin; (B) protein expression related to pyroptosis measued by western blotting.

150x61mm (300 x 300 DPI)

Appendix B

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 corrcetion.

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 espresented 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 tredency

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 levlels

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

Appendix C

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., Itd (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:

*Author for correspondence: lhaojianhuhenhao@163.com and sunwei_223@163.com)
† These authors contributed equally to this work
Present address: College of Agriculture, Tongren Polytechnic College, Bijiang District, Tongren City, Guizhou, 554300, China

1.Introduction

Pseudorabies virus (PRV) or suid a herpesvirus 1 is the pathogen of porcine Aujeszky's disease (AD), . which causes the respiratory system, nervous system and . reproductive systems[1]. Many mammals, including pigs, are susceptible to PRV infection, such as cattle, sheep, rabbits, cats, dogs, guinea pigs, rats and . mice[2]. However, pigs are the only susceptible animals that can survive, although the prognosis of the disease largely depends on the factors including inoculation site, virus strain and . titer as , well as the age of pigs[3]. AD is a highly infectious disease with high mortality in piglets. Transmission mainly occurs through direct contact with oral and nasal secretions but can also occur through aerosol and the placenta or sexual intercourse[1]. 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. However, they cannot eradicate the disease because most of them . can prevent virus potential infection and reactivation and shedding of virulent . byld 141. Due to the impact of AD on the pig industry, some countries are trying to eradicate AD based on the DIVA(Differentiating Infected From Vaccinated animals) program. However, since 2011, the outbreak of AD occurred in pigs vaccinated with PR vaccine in China, which indicates that the AD vaccine can not provide effective treatment to prevent wild PR infection[3].

Mice and rabbits are usually used to study PRV in the laboratory. After infection, animals showed abnormal excitement and nasal itching, accompanied by convulsions and rapid death. In mice, PRV almost manifested as a neurogenic infection of the central nervous system(CNS), accompanied by fulminant central nervous symptoms and high mortality[4]. PRV is known to cause severe encephalitis in piglets, various non-native hosts, even in humans[5]. few studies have focused on the pathogenesis of encephalitis. It is known that pyroptosis is involved in the immune response in various types of cells, which can be triggered by a variety of . pathological stimuli, leading to the secretion of pro-inflammatory cytokines and intracellular content[6]. Inflammation is a double-edged sword, which has a crucial role in metabolism. A mild inflammatory response could protect the body to a certain degree, help to repair damaged tissue, and be beneficial to steady-state reconstruction. Nevertheless, excessive inflammation may form "cytokine storms", . leading to tissue damage. In the present work, we describe the influence of PRV on the immune factor and pyroptosis-related factor . in mice brains.

Annexin V-FITC/PI Apoptosis Kit was obtained from BD 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 Boss Company (Beijing, China). Caspase-1antibody was obtained from Bioseo Biological Technology com,Ltd (Wuhan, Hubei, China). PrimeScript RT reagent Kit was bought from ., TAKARA company (Dalian, China). PRV-HLJ strain (MK088279.1) isolated from Heilong Jiang was provided by Professor Jinglei Wang .. from Harbin Veterinary Research Institute, CAAS. Eighty 6-week-old female BalbVC mice were obtained from Dossy Experimental Animal Corporation (Chengdu, China).

2.2 Experimental

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 96 lgci ., 48 lgci ., 60 lgci ., and . 72 lgci .. The mice in the control group were injected with 0.2 mL of normal saline (NS) by subcutaneous inoculation on the back. The mice in another group were received 0.2 mL PRV-HLJ strain (104 TCID50/100 μL) at the same inoculation site. The animals were fed in the room illuminated with a 12 h light-dark cycle. The ambient temperature and relative humidity were maintained at 22-24℃ and 48-68%, respectively. Ten mice were fed in each cage and given the above dosage. Water and diet was .. provided ad libitum. Mice brain tissues in each group were collected aseptically. Subsection specimens were snap frozen ., and stored at -86℃ for RNA extraction. In addition, portions of the brains were fixed in 4% paraformaldehyde solution for histopathological examination.

2.3 Histopathological analysis

The 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% per cent . or .. absolute alcohol for dehydration. After that, processed .. in paraffin embedding routine. The paraffin-embedded tissue was sliced into a five μm section, dewaxed in xylene and .. then rehydrated in graded alcohols. The section was stained with hematoxylin-eosin (H.E) staining and then examined under a light microscope for histopathological examination.

2.4 Detection of pyroptosis by flow cytometry in brain

The rate of pyroptosis cells in the brain was measured using an annexin V-FITC/PI Apoptosis kit according to the instructions provided. Brains were taken from mice, which were humanely killed at a time above mentioned .., ground to form a suspension and .. filtered with a 100-mesh nylon screen. The cells were washed three times with pre-cold PBS and adjusted at a concentration of 1×106 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 a dark atmosphere. Each tube was added with 300 μL of binding buffer and then detected with an 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 an "Animal Total RNA Isolation Kit" according to the kit instructions. 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 was 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 the brain. The 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 polyvinylidene difluoride (PVDF) membrane. Then, the PVDF membrane was blocked with TBST solution contained with 5% non-fat milk at room condition for 2 ..h and then incubated at 4℃ condition for 12 h with the corresponding primary antibodies diluted with a solution:NLRP3 ,(1:1000), Caspase-1(1:800), GSDMD(1:500), IL-1β(1:500), IL-18(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 for data analyses.The . results were presented as mean ± standards deviations (mean±SD). One-way analysis of variance (ANOVA)

package in SPSS 22.0 was used to evaluate the statistical significance between PRV and control group. GraphPad Prism6.0 software was used to perform statistical artworks ... In all statistical comparisons, the p-value was introduced as a judgment of the statistical difference.

3.Results

3.1 Clinical symptoms and histopathological analysis

At 36 hpi ... the mice infected with PRV generally appeared typical ... clinical signs, including depression, anorexia and ... encephalic itch. Mortality occurred within the period of 48-72 hpi ... However, none of the mice in the 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 group (Figure 1). Normal ... brain structure was found in the control and PRV-inoculated mice before 36 hpi ... (Figure 1A and 1b). At 48 hpi ..., hyperemia appeared in brain tissue, followed by perivascular space widened, perivascular lymphocytes increased, as well as degeneration and necrosis occurred to some neurons (Figure 1C). Focal inflammatory cellular infiltration was found in the brain in 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. Interferon (IFN) is an antiviral molecule, which has a pivotal role in the clearance of invading pathogenic microorganisms[9]. In this work, the transcriptional levels of IFN-α, IFN-β and ... IFN-γ were measured 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 from 36 hpi ... ($p<0.01$) and peaked at 48 hpi ... ($p<0.01$) and then down-regulated to 60-72 hpi ... ($p<0.05$, Figure 2A-B). ... Furthermore, IFN-γ expression in brain ... was down-regulated before 36 hpi ... and then up-regulated until 60 hpi ... ($p<0.01$, 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 the brain were remarkably up-regulated caused by PRV infection and peaked at 60 hpi ... ($p<0.01$) followed by down-regulated at 72 hpi ... (Figure 2 D and F). After PRV infection, the IL-1β expression in the brain was up-regulated from 36 hpi ... and lasted for the whole experiment period ($p<0.01$, Figure 2G). In addition, IL-18 expression was up-regulated from 36 hpi ... and had the same tendency as IL-1β ($p<0.01$, Figure 2G). The cytokine storm caused by IFN-α, IFN-β, TNF-α, IL-1β, IL-6 and ... IL-18 were 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 ... ($p<0.01$) and 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 pyroptosis cells in the brain

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

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

To further investigate the effect of PRV on the pyroptosis in vivo, the proteins involved in pyroptosis ... signal pathway were detected by blotting, including NLRP3, Caspase-1, GSDMD, IL-1β and ... IL-18. From the result in figure 5, NRBP3 decreased at 36 hpi ... ... followed by an increasing tendency in a time-dependent manner. In addition, the Caspase-1 level was significantly up-regulated by PRV from 36 hpi ... and maintained at a high level until the end of this experiment by comparing it with that in the control group. Furthermore, GSDMD, a pyroptosis marker protein, was also measured in all groups. As shown in Figure 5A, the amount of GSDMD was higher in all PRV groups than that in ... the control ... In addition, the expression levels of IL-1β and IL-18, two cytokines related to pyroptosis, were determined by blotting. The result demonstrated that PRV could elevate the protein levels of IL-1β and IL-18.

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

The mRNA expression levels of pyroptosis-related factors were further detected by qRT-PCR in this research. As shown in Table S3, the mRNA expression level of NRBP3 were ... markedly increased ($p<0.01$) in a time-dependent manner following PRV treatment. Moreover, the mRNA expression levels of Caspase-1 in the PRV inoculation groups were also up-regulated ($p<0.01$) when compared that ... that among groups. Besides, the GSDMD mRNA expression level was up-regulated in an increased tendency ($p<0.01$) compared with that in the control group.

3.Discussion

PRV is a kind of neurophilic α-herpesvirus, which belongs 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 the lab, after intranasal infection in adult mice, PRV enters peripheral nerve cells and spreads to the central nervous system[10]. Previous studies on PRV mainly focused on pathogenicity and the resulting host immune response. The aim of this research was ... to describe the kinetics of cytokine secretion in vivo and to clarify whether cytokine storm is involved in the pathogenesis of PRV. In this work, we identified cytokine storm and pyroptosis in the brain ... causes of rapid death in mice infected with PRV.

The innate immune system is the first line in defending against the invasion of pathogens, accompanied by the recruitment of immune cells and inflammatory response[11]. Inflammation is a process to solve microbial infection and a complex process involving the regulation of cytokine production. Dysfunction of these factors can induce cytokine storms and related multiple organ failure[12]. An inflammatory response is usually caused by various of ... pro-inflammatory cytokines, such as TNF, IL-1 and ... IL-6. These cytokines are the kind of pleiotropic proteins, involves ... the regulation of cell death in inflammatory tissue, vascular endothelial cell permeability, attracting the blood cells to inflammatory tissue, and acute phase ... protein production[13].

Cytokines play an important ... role in all items of the immune response, coordinating the innate and adaptive immune response. Consequently, in most cases, cytokines play a protective role in restoring 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[14]. 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 protective[15]. For example, over expression ... of TNF-α, IL-1, and IL-6 in the immune system could lead to vascular leakage, systemic fatigue, cardiomyopathy, and acute phase ... protein synthesis[15]. In addition, persistent excessive IFN-α/β may also be harmful to the immune system[16]. In the present study, we found that 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 pro-inflammatory factors compared to that in the control group ($p<0.01$). This storm was consistent with the histopathological changes including ... hyperemia and inflammatory cell infiltration in the mice brain. Studies have shown that PRV could regulate the expressions of cytokines, including type I and type II interferon and inflammatory factors, to establish a successful infection[16,17]. Furthermore, IFN-α and IFN-β mediate a positive feedback regulation by binding to IFN-α and IFN-β receptor in an autocrine or paracrine manner[18].

PRV infection can induce apoptosis, which has been reported previously in vitro and vivo ...[19,20]. 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[21]. The discrepancy was found in this research may be induced by a new kind of PCD in the 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[22]. In this research, an increasing tendency of pyroptotic cells induced by PRV were ... detected in the front through flow cytometry by comparing with the control group ($p<0.01$). Furthermore ..., the expression of genes and proteins related to pyroptosis ... pathway were elevated by qRT-PCR and blotting methods, respectively. Inflammasomes are cytosolic sensors that could activate caspase-1[23]. Once activated, caspase-1 can process and maturates IL-1β and IL-18 precursors, as well as cleave GSDMD, resulting in cell membrane channel opening and the pyroptosis -[24]. Among the inflammasomes, NLRP3 is currently the most well-known one, which response ... to various stimuli. NLRP3 was activated by PRV ... in this research. In addition, GSDMD, a key executor in pyroptosis[24], also was activated by PRV. This activity of GSDMD leads to the indirect release of IL-1β and IL-18 from membrane pores[25]. The pyroptotic cell-line decision provides a large amount of inflammatory response at the site of infection. This ... was consistent 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 brains. 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 detailed information about the pathogenesis to pig and other mammal animals need to be further clarified in the future.

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