L-serine lowers the inflammatory responses during *Pasteurella multocida* infection

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ABSTRACT

*Pasteurella multocida* causes a variety of infectious diseases in various species of mammals and birds, resulting in enormous economic loss to the modern livestock and poultry industry. However, the mechanism of host-pathogen interactions is unclear. Here we found that L-serine levels were significantly decreased in murine lungs infected with *P. multocida*. Exogenous L-serine supplementation significantly increased the survival rate of mice, and decreased the colonization of *P. multocida* in the lungs of mice. Notably, L-serine decreased the macrophage- and neutrophil-mediated inflammatory responses in mice during *P. multocida* infection.

KEYWORDS: Inflammation; L-serine; Macrophage; Neutrophil; *Pasteurella multocida*
INTRODUCTION

*Pasteurella multocida* (*P. multocida*) is a Gram-negative bacterium and primarily causes hemorrhagic septicemia and pulmonary inflammation in poultry and livestock (1-5). *P. multocida* is divided into five different serotypes, including A, B, D, E and F, based on the different capsular antigen (6, 7). *P. multocida* serotype A always triggers bovine respiratory diseases with a high morbidity, mainly resulting in significant bovine pulmonary tissue lesions (8-11). Unfortunately, the pathogenesis and the host-pathogen interaction remain to be fully understood; and no effective methods are available to prevent and/or treat *P. multocida* infection.

Increasing studies have found that glucose, fatty acid and amino acid metabolism play important roles in the pathogenesis of *P. multocida* (12-16). Notably, our previous investigations have shown that amino acids shape the pathogenesis of *P. multocida* infection. For example, supplementation with glutamine has beneficial effects against *P. multocida* infection in mice pre-immunized with the inactivated *P. multocida* vaccine by enhancing general defense responses and decreasing expression of specific virulence factors (17). Furthermore, dietary proline or arginine supplementation enhances immune responses through increasing serum antibody titer and glutathione peroxidase (GSH-PX) level, and decreasing the production of cytokines (e.g., IL-6, IL-8, and TNF-α) in inactivated *P. multocida* vaccine-immunized mice (18, 19). Based on the above investigations, we speculated that amino acid metabolism plays important roles in pathogenesis of *P. multocida* infection. Therefore, this study was conducted to explore the interaction between host
and *P. multocida* from the perspective of amino acid metabolism.

Multiple lines of investigations have discovered a link between serine and immune cell function, and even infection (20). For example, serine metabolism shapes the fate decision of immune cells, like T cells and macrophages, though one-carbon metabolism (21, 22) and glutathione (GSH) synthesis (23). However, the role of serine in immune responses during *P. multocida* infection is unknown. Here, we found that *P. multocida* infection remarkably shapes serine metabolism in the mouse lung. Notably, exogenous L-serine administration lowers bacterial colonization, and macrophage and neutrophil-mediated inflammation, as well as enhances the survival rate in mice during *P. multocida* infection.
RESULTS

Serine metabolism changes during *P. multocida* infection. Our previous study has explored the differential expressed genes (DEGs) during *P. multocida* infection in mice (9). Transcriptomic analysis identified DEGs from 16 amino acid biosynthesis pathways (Supplementary table 1). Further analysis of these pathways suggested that L-serine, glycine and threonine metabolism (path:mmu00260) (Fig. 1A), and arginine biosynthesis and metabolism (path:mmu00330) (Fig.1B) were obviously enriched. The changes of DEGs in path:mmu00260 and path:mmu00330 from transcriptomic analysis (Fig. 1C) were also validated by qRT-PCR at 16 h post infection (Fig. 1D), although there was no significant difference at 4 h (Supplementary Fig. 1A) and 8 h (Supplementary Fig. 1B) post infection. Notably, *P. multocida* infection enhanced the expression of *Psat1, Phgdh, Pgaml, Shmt1, Shmt2, Gnmt* and *Gar* (Fig. 1A, C and D), suggesting that *P. multocida* infection promotes L-serine and glycine metabolism.

In order to further validate the changes of amino acids during *P. multocida* infection, we determined the concentrations of free amino acids in mouse lung by L8900 amino acids analyzer. Levels of 14 amino acids showed significant differences after infection, and of which 10 amino acids decreased (Ser, Gly, Thr, Arg, Pro, Tyr, Met, Leu, Lys, and Orn) (Fig. 1E). Collectively, these results indicate that *P. multocida* infection induces significant changes of amino acid metabolism, especially about L-serine, glycine and threonine metabolism.

Exogenous L-serine supplementation enhances resistance to *P. multocida* infection. To further explore the potential effects of L-serine on mice infected with *P.
multocida, we supplemented L-serine to mice before infection. We supplemented the L-serine to the mice through intramuscular injection with 0.2 mg/kg. L-serine supplementation lowered the bacteria colonization in the lungs, TNF-α level in the lung, as well as IL-1β and IFN-γ levels in the serum during *P. multocida* infection (Supplementary Fig. 2). We then supplemented L-serine before infection through intranasal administration of serine with dosage of 0.2 mg/kg because previous study found that the drug is more effective when it was administered directly to lung tissue through trachea injection than other methods (24). Serine lowered the bacteria colonization in the lungs and inflammatory cytokine production at 8 h and 16 post infection (Supplementary Fig. 3).

Notably, the survival rate of mice infected with *P. multocida* was significantly increased by intranasal administration with 2 g/kg L-serine (Fig. 2A). The bacteria colonization in the lung was decreased at 4 h, 8 h, and 16 post infection (Fig. 2B). Intranasal administration of serine significantly increased the lung levels of free L-serine, but not others, at 8h post infection (Fig. 2C, Supplementary Fig.4A). Serine supplementation inhibited the mRNA expressions and secretion of IL-1β, IL-17, IFN-γ and TNF-α in the lungs and serum at 4 (Fig. 2D-F), 8 (Fig. 2G-I) and 16 (Fig. 2K-M) h post infection, with the most significant changes at 8 and 16 h post infection. H&E staining also showed that intranasal administration of L-serine alleviated the infection-induced pneumonia at 8 h post infection (Fig. J). Interestingly, intranasal administration of serine did not change the levels of serine in the lung at 16 h post infection (Supplementary Fig.4B). The possible reason may be that serine...
supplementation significantly increased expressions of L-serine metabolism-related enzymes, including Shmt1 and Phgdh, at 16 h post infection, but not at 4 and 8 h post infection (Supplementary Fig.5).

Above data indicated that L-serine reduces the bacterial load of P. multocida and the inflammatory responses during infection. In order to explore whether these effects depend on its direct inhibition on P. multocida growth, the influence of L-serine (even glycine and threonine) on the growth of P. multocida in vitro was explored. Different concentrations of L-serine, glycine and threonine were added to culture medium for P. multocida. The results showed that different concentrations of L-serine, glycine and threonine did not directly affect the growth of P. multocida in Martin broth medium, and even in a nutrition defect medium-LB medium (Supplementary Fig.6A-C). Also, L-serine, glycine and threonine did not affect the phagocytosis of P. multocida by macrophages, and its adhesion properties to macrophages and epithelial cells (Supplementary Fig.6D-E).

Collectively, these results implicate that L-serine shows beneficial effects during P. multocida infection, including lower death rate, bacterial load and inflammation in the lung.

L-serine inhibits macrophage and neutrophil-mediated inflammatory responses. To determine whether L-serine administration inhibits host inflammatory responses by regulating the functions of alveolar macrophages, the alveolar macrophages in vivo were deleted using clodronate-loaded liposome. The deletion of alveolar macrophages was confirmed by immunoblotting for CD68 (Fig.3A-D). In this model, serine
supplementation decreased the secretion of inflammatory cytokines, including IL-1β, IL-17, IFN-γ and TNF-α both in the lung (Fig.3E) and serum (Fig.3F) during *P. multocida* infection. The possible reason is that serine has no effect on alveolar macrophage-mediated inflammation during *P. multocida* infection or there is compensative increase in neutrophils in this model. Interestingly, there was no change about the secretion of IL-1β, IL-17, IFN-γ and TNF-α in the lung (Fig.3E), and IL-1β, IL17 and IFN-γ in the serum (Fig.3F) after alveolar macrophage clearance, suggesting a compensative increase in these cytokines-producing cells in the lung after macrophage clearance. Notably, there were significantly increased numbers of neutrophils after macrophage deletion (Fig.3G-J).

To verify whether L-serine administration alleviates inflammation through modulating the functions of neutrophils, anti-Ly6G monoclonal antibodies were then used to eliminate neutrophils. Neutrophil elimination was confirmed by immunoblotting for Ly6G (Fig.4A-D). Similar to the observations in macrophage-deleted models, serine supplementation significantly decreased the secretions of IL-1β, IL-17, IFN-γ and TNF-α in the lung (Fig.4E) and serum (Fig.4F). Interestingly, the numbers of macrophages markedly increased after neutrophil elimination (Fig.4G-J).

Thus, clodronate-loaded liposome and anti-Ly6G monoclonal antibody were used simultaneously to eliminate macrophages and neutrophils. Our results showed that about 90% of macrophages and neutrophils were eliminated (Fig.5A-H). Although, serine supplementation lowered the bacterial colonization in the lung without
treatment of clodronate-loaded liposome and anti-Ly6G monoclonal antibody, serine supplementation failed to influence the bacterial colonization in the lung (Fig. 5I) in macrophage and neutrophil-cleared model. More importantly, serine supplementation failed to affect the secretion of inflammatory cytokines in the lung (Fig. 5J) and serum (Fig. 5K) in macrophage and neutrophil-cleared model. Taken together, we conclude that L-serine reduces inflammatory responses mediated by macrophages and neutrophils during *P. multocida* infection.
Discussion

Knowledge about amino acid metabolic cross talk between a pathogen and its host has advanced in recent years, including: 1) the host has the ability to alter amino acid metabolism after an infection; 2) amino acids affect host immune responses against a pathogen; 3) amino acids play an important role in the physiology and virulence of pathogens (25). There are significant changes about the levels of amino acids during *P. multocida* infection. Notably, we found that *P. multocida* infection shapes the serine, glycine and threonine metabolism, and lowers the levels of glycine, threonine and serine. This raises an interesting question about the underlying mechanism for the down-regulated L-serine during *P. multocida* infection. We have showed that the enzymes related to L-serine metabolism are significantly up-regulated during *P. multocida* infection. Apart from the possibility of increase in serine metabolism, whether the synthesis and transportation of L-serine is blocked during *P. multocida* infection remain to be further investigated. Besides serine, it is of worthy to explore the metabolic talk between *P. multocida* and host on other amino acids.

Indeed, previous studies have found that exogenous addition of arginine, glutamine and/or proline highly shapes the pathogenesis of *P. multocida* infection (17-19).

In addition to their nutritional functions, amino acids also play important roles in the modulation of the inflammatory responses during bacterial infection (26, 27). In this study, serine inhibits macrophage and neutrophil mediated inflammatory responses. Besides macrophages and neutrophils, other immune cells are also involved in the pathogenesis of *P. multocida* infection, like effector T cells (9),
dendritic cells (28), and even B cells (29). Thus, it is interesting to know whether serine affects the functions of other immune cells, such as T cells, B cells and dendritic cells, during *P. multocida* infection. As we have found that depletion of macrophages and neutrophils increases the bacterial burden in the lung, and this depletion cannot fully block the cytokine production, thus, it is interesting to uncover the influence of serine on functions of other immune cells, especially Th1 cells and Th17 cells, during *P. multocida* infection.

Macrophages are the first line of defense against host pathogen invasion (30). Notably, inflammasomes, including NALP1, NLRP3, NLRC4 and AIM2, are essential for regulating the inflammatory responses in macrophages (31) by activating the pro-inflammatory protease caspase-1 to promote the maturation and secretion of IL-1β, and by inducing apoptosis (32-34). IKK/NF-κB signaling pathways involve in the regulation of the inflammatory responses (35), and NF-κB is a key transcriptional regulator of the macrophages (36). mTOR is a central metabolic pathway that couples nutrient sensing to the regulation of metabolic processes, and affects macrophage polarization (37, 38). It is interesting to explore the underlying mechanisms by which serine inhibits the macrophage polarization, like the activation of inflammasome, NF-κB and mTOR signaling.

It is well known that neutrophils have the same important roles as macrophages in the participation of the inflammatory responses (39-41). Although macrophages and neutrophils have phagocytic and bactericidal functions (42-46), the main function of neutrophils is non-specific defense (innate immunity) (47), while macrophages are...
involved in innate immunity and specific defense (cellular immunity) (48).

Furthermore, in macrophages, enhanced pro-IL-1β processing is dependent on caspase-1, however, in neutrophils, the secretion of IL-1β is dependent on serine proteases (49-51). In this study, the results showed that L-serine has immunosuppressive effects on macrophages and neutrophils, however, whether the underlying mechanism for the immunosuppressive effect of serine on neutrophils is consistent with those in macrophages still needs to be revealed.

Another interesting observation is that L-serine reduces the colonization of *P. multocida* in mice. Notably, serine does not directly affect the growth and adhesion of *P. multocida*. The possible explanation is that serine inhibits the production of inflammatory cytokines, resulting in lower bacterial load of *P. multocida*. Previous study has found that the bacterial load of *L. monocytogenes* in the mouse liver is associated with the production of inflammatory cytokines (e.g., IL-18), and administration of IL-18 promotes the load of *L. monocytogenes*(52). And another study found that ablating inflammatory monocytes or impairing their recruitment to the lungs improves murine survival and reduces fungal proliferation and dissemination(53). Similarly, a study found that clearance of neutrophils enhances mouse survival and reduces bacterial colonization and inflammation induced by *Ehrlichia chaffeensis* (54).

This experiment is based on mouse model, however, the *P. multocida* used is mainly isolated from cattle and mainly caused infection in cattle. Therefore, further experimental exploration is needed, including whether serine is resistant to infection.
by other sources (e.g. poultry and pig) or different serotype strains (e.g. B, D, E, and F), and whether serine has anti-infective effects on different hosts (e.g. bovine, birds, or human).

In conclusion, we found that L-serine levels in the lungs of mice infected with *P. multocida* are significantly down-regulated. Notably, exogenous L-serine administration lowers bacterial colonization, macrophage and neutrophil-mediated inflammation, as well as enhances the survival rate in mice infected by *P. multocida*. Based on this study, L-serine can be considered as a nutrient additive for the prevention of macrophage-associated diseases (e.g. inflammation, bacterial infection, and tissue damage) in animals and/or humans.
Materials and methods

**Bacterial strains.** Bovine Pasteurella multocida serotype A strain CQ2 (PmCQ2, GenBank accession number: LIUN00000000), a highly virulent strain (Intramuscular LD50 = $2.2 \times 10^5$ CFU in mice) (55), which is isolated from a bovine lung. PmCQ2 was generally grown in Martin’s broth agar containing 5% horse serum at 37°C for 24 h (17).

**Mice.** Female C57BL/6 mice (weight 18-22 g, 6-8 weeks old) were purchased from Laboratory Animal Center of Third Military Medical University (Chongqing, China) and housed in individually ventilated, pathogen-free cages (temperature at 20–30°C, relative humidity at 50-60%, lighting cycle at 12 h/day) with free access to food and water. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Laboratory Animal Ethical Commission of the Southwest University (Permit No. 11-1025), Chongqing, China.

**P. multocida infection in mice.** Mice were infected by an intraperitoneal injection with *P. multocida* at the dose of $2.2\times10^5$ CFU (LD50) in 100 μL. In the control group, mice (gender and age matched) were injected intraperitoneally with equal dose of saline. Total 556 mice were used in this study. Survival rates (n =10) were measured in both groups after injection. Mice were also euthanized for collection of tissues and serum samples at 4h (n =6), 8h (n =6), and 16 h (n =10) post-infection.

**Bacterial colonization.** To measure the bacterial load, the lung tissues of mice (n=6 or 10) were collected at different time point post-bacterial infection. The tissues were homogenized aseptically and bacterial loads were quantified by 10-fold serial dilution.
in saline. These different dilutions were plated in triplicate on Martin's broth agar and were incubated at 37°C up to 24 h to count CFU.

**Quantitative Real-Time-PCR (qRT-PCR).** The lung tissue was quickly collected and stored in liquid nitrogen. Total RNA of the lungs were acquired as described previously (56). cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Specific primers were listed in (Supplementary Table S2). qRT-PCR was performed according to previous study (9).

**Enzyme Linked Immunosorbent Assay (ELISA).** Lung homogenates were freezing and thawing (frozen in liquid nitrogen for 5 min and then melted on ice) three times. After centrifugation at 12,000 rpm for 10 min at 4°C, supernatant was acquired. Cytokines (e.g., IL-17, IL-6, and IFN-γ) were detected in the supernatant or the serum with ELISA kits in accordance with the manufacturer's protocol. ELISA kit for cytokine were purchased from eBioscience, USA.

**Lung amino acid analysis.** Lung amino acids were analyzed with isotope dilution liquid chromatography-mass spectrometry methods as previous study(57).

**The clearance of alveolar macrophages in mice.** Macrophages clearance was assessed refer to the method described in the previous literature (58, 59). Briefly, the mice were completely anesthetized intraperitoneal with 80ul 1.5% pentobarbital sodium (Beijing huaye world chemical co. LTD). Then 200 ml empty liposomes or liposome chlorophosphonate (LIPOSOMA) was administered through the trachea into the mouse lung.

**The deletion of neutrophils in mice.** Neutrophil clearance was conducted based on...
the method described in the previous literature (60). Briefly, mice were treated with 300 mg anti-mouse Ly6G (clone1A8) antibodies (Bio-X-cell) by intraperitoneal injection. Antibodies were administered every 2 days up to 4 times.

**Histopathological Examination and Immune-histochemical (IHC) staining of tissue section.** The histopathological examination experiments were performed as described in the previous study (9). The IHC experiment is conducted as described in the previous study (57). The fixed slices were dehydrated in graded ethanol, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histopathological examinations. For the lung immunohistochemical staining of CD68 and Ly6g, deparaffinized lung sections were treated with 3% H2O2 in methanol for 30 min to block endogenous peroxidase, and tissue sections were boiled in 0.01 M citrate buffer for antigen retrieval and then blocked with 1% normal goat serum (Southern Biotech, AL, USA) for 30 min at room temperature. Sections were then incubated overnight at 4°C with anti-CD68 antibody (Proteintech, China, 1:500 dilution) or anti-Ly6g antibody (Abcam, United Kingdom, 1:50 dilution). After washing in PBS, the sections were visualized by biotinylated secondary antibodies followed by incubation with HRP-Conjugated Streptavidin for 30 min (R&D Systems, London, UK). Then, sections were incubated with 3,3-diaminobenzidine (DAB; Aladdin®, Shanghai, China) for 3 minutes. After washing in PBS, then all specimens were lightly counterstained with hematoxylin for 5 min. The areas of CD68-positive (+) or Ly6G-positive (+) lung cells, as well as total areas of lung sections were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD,
USA). CD68+ or Ly6G+ cells were examined in a total of 10 fields at 40x10 magnification per animal. The data collectors were unaware of the treatment status of the examined slides.

**Phagocytosis and adhesion assay of macrophage and lung epithelial cells.**

Peritoneal macrophages were isolated from mice as previously described (24, 61). Peritoneal macrophages and lung epithelial cells were cultured in RPMI 1640 medium (Gibco, USA) with 10% heat-inactivated FBS (Gibco, USA) and counted with a hemocytometer, then incubated overnight at 37°C with 5% CO₂ in 48-well microplates at a density of 2x10⁵ cells/well. Then cells were washed with PBS to remove the non-adherent cells. The adherent cells were cultured in RPMI 1640 medium supplemented with 10 mM L-serine, threonine or glycine for 2 h, subsequently challenged with *P. multocida* at an MOI of 1 for 16 h. Cells were washed three times with chilled PBS to remove non-associated bacteria and lysed in PBS containing 0.1% Triton X-100. The cell lysates were diluted with PBS and grown on Martin’s agar plates at 37 °C for 18-24 h to determine the number of *P. Multocida* (total number of adhered and phagocytosed *P. Multocida*). The cells of another 48-well microplates were treated with ciprofloxacin (100 mg/ml) for 30 min, washed 3 times with PBS to remove extracellular ciprofloxacin. Then cells were lysed by addition of 0.1% (v/v) Triton-X 100 in PBS to count numbers of *P. Multocida* (number of phagocytosed *P. Multocida*) (11). The number of adhered *P. Multocida* = total number of adhered and phagocytosed *P. Multocida* - number of phagocytosed *P. Multocida*. 

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**Statistical analyses.** Data shown are the means ± the standard error of the mean (SEM). Data were statistically analyzed according to our previous papers (62, 63).

Data between two groups were analyzed by unpaired t test (Prism 6.0) if the data are in Gaussian distribution and have equal variance, or by unpaired t test with Welch’s correction (Prism 6.0) if the data are in Gaussian distribution but show unequal variance, or by non-parametric test (Mann-Whitney U test, Prism 6.0) if the data are not normally distributed. The Gaussian distribution of data was analyzed by D’Agootino-Pearson omnibus normality test (Prism 6.0) and Kolmogorov-Smirnov test (Prism 6.0). The variance of data was analyzed by Brown-Forsythe test (Prism 6.0). Differences with P<0.05 were considered significant.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

**Authors Contributions**

WR and YP designed the experiment; FH, CW, YX, PL, HZ and YY conducted the
experiment; ZY, NL and GZ helped with data analysis and figures; FH, YX and MW drafted the manuscript; WR revised and approved the final manuscript.
References

1. Patel SJ, Joshi D, Raval S, Patel BJ, Patel J, Chauhan H, Chandel BS, Patel BK, Shah NM. 2016. Clinicopathological studies of Pasteurella multocida B: 2 experimental infection in rabbits. Indian J Anim Sci 86:380-386.

2. Bhat P, Singh ND, Leishangthem GD, Kaur A, Mahajan V, Banga HS, Brar RS. 2016. Histopathological and immunohistochemical approaches for the diagnosis of Pasteurlosis in swine population of Punjab. Veterinary world 9:989.

3. Zhao X, Liu Q, Xiao K, Hu Y, Liu X, Li Y, Kong Q. 2016. Identification of the crp gene in avian Pasteurella multocida and evaluation of the effects of crp deletion on its phenotype, virulence and immunogenicity. BMC Microbiol 16:125.

4. Haji S. 2015. Pasteurlosis in sheep and its drug susceptibility pattern in Mojo district, East Shoa ZoneAddis Ababa University, N1 - 2019-01-09 16:26:00.

5. Xiao K, Liu Q, Liu X, Hu Y, Zhao X, Kong Q. 2015. Identification of the Avian Pasteurella multocida phoP Gene and Evaluation of the Effects of phoP Deletion on Virulence and Immunogenicity. Int J Mol Sci 17.

6. Carter GR. 1955. Studies on Pasteurella multocida. I. A hemagglutination test for the identification of serological types. Am J Vet Res 16:481-4.

7. Du H, Fang R. 2016. Comparative Genomics Analysis of Two Different Virulent Bovine Pasteurella multocida Isolates. 2016:4512493.
8. Dabo SM, Taylor JD, Confer AW. 2007. Pasteurella multocida and bovine respiratory disease. Anim Health Res Rev 8:129-50.

9. Wu C, Qin X, Li P, Pan T, Ren W, Li N, Peng Y. 2017. Transcriptomic Analysis on Responses of Murine Lungs to Pasteurella multocida Infection. Front Cell Infect Microbiol 7:251.

10. Li N, Long Q, Du H, Zhang J, Pan T, Wu C, Lei G, Peng Y, Hardwidge PR. 2016. High and low-virulent bovine Pasteurella multocida capsular type A isolates exhibit different virulence gene expression patterns in vitro and in vivo. Vet Microbiol 196:44-49.

11. Fang R, Du H, Lei G, Liu Y, Feng S, Ye C, Li N, Peng Y. 2019. NLRP3 inflammasome plays an important role in caspase-1 activation and IL-1beta secretion in macrophages infected with Pasteurella multocida. Vet Microbiol 231:207-213.

12. Wannemacher RW, Jr., Beall FA, Canonico PG, Dinterman RE, Hadick CL, Neufeld HA. 1980. Glucose and alanine metabolism during bacterial infections in rats and rhesus monkeys. Metabolism 29:201-12.

13. Smith CL, Dickinson P, Forster T, Craigon M, Ross A, Khondoker MR, France R, Ivens A, Lynn DJ, Orme J, Jackson A, Lacaze P, Flanagan KL, Stenson BJ, Ghazal P. 2014. Identification of a human neonatal immune-metabolic network associated with bacterial infection. Nat Commun 5:4649.

14. Wils-Plotz EL, Jenkins MC, Dilger RN. 2013. Modulation of the intestinal environment, innate immune response, and barrier function by dietary
threonine and purified fiber during a coccidiosis challenge in broiler chicks. Poult Sci 92:735-45.

15. Sun X, Song L, Feng S, Li L, Yu H, Wang Q, Wang X, Hou Z, Li X, Li Y, Zhang Q, Li K, Cui C, Wu J, Qin Z, Wu Q, Chen H. 2018. Fatty Acid Metabolism is Associated With Disease Severity After H7N9 Infection. EBioMedicine 33:218-229.

16. Dong Y-W, Jiang W-D, Liu Y, Wu P, Jiang J, Kuang S-Y, Tang L, Tang W-N, Zhang Y-A, Zhou X-Q. 2017. Threonine deficiency decreased intestinal immunity and aggravated inflammation associated with NF-κB and target of rapamycin signalling pathways in juvenile grass carp (Ctenopharyngodon idella) after infection with Aeromonas hydrophila. British Journal of Nutrition 118:92-108.

17. Chen S, Liu S, Zhang F, Ren W, Li N, Yin J, Duan J, Peng Y, Liu G, Yin Y, Wu G. 2014. Effects of dietary L-glutamine supplementation on specific and general defense responses in mice immunized with inactivated Pasteurella multocida vaccine. Amino Acids 46:2365-75.

18. Ren W, Zou L, Ruan Z, Li N, Wang Y, Peng Y, Liu G, Yin Y, Li T, Hou Y, Wu G. 2013. Dietary L-proline supplementation confers immunostimulatory effects on inactivated Pasteurella multocida vaccine immunized mice. Amino Acids 45:555-61.

19. Wenkai R, Lingxiu Z, Nengzhang L, Yan W, Gang L, Yuanyi P, Jiannan D, Lichuang C, Yulong Y, Guoyao W. 2013. Dietary arginine supplementation
enhances immune responses to inactivated Pasteurella multocida vaccination in mice. Br J Nutr 109:867-872.

20. Cheng ZX, Guo C. 2019. Glycine, serine and threonine metabolism confounds efficacy of complement-mediated killing. 10:3325.

21. Ma EH, Bantug G, Griss T, Condotta S, Johnson RM, Samborska B, Mainolfi N, Suri V, Frencla B, Verway MJ, Raissi TC, Tsui H, Boukhaled G, Henriques da Costa S, Frezza C, Krawczyk CM, Friedman A, Manfredi M, Richer MJ, Hess C, Jones RG. 2017. Serine Is an Essential Metabolite for Effector T Cell Expansion. Cell Metab 25:345-357.

22. Yu W, Wang Z, Zhang K, Chi Z, Xu T, Jiang D, Chen S, Li W, Yang X, Zhang X, Wu Y, Wang D. 2019. One-Carbon Metabolism Supports S-Adenosylmethionine and Histone Methylation to Drive Inflammatory Macrophages. Mol Cell doi:10.1016/j.molcel.2019.06.039.

23. Rodriguez AE, Ducker GS, Billingham LK, Martinez CA, Mainolfi N, Suri V, Friedman A, Manfredi MG, Weinberg SE, Rabinowitz JD, Chandel NS. 2019. Serine Metabolism Supports Macrophage IL-1beta Production. Cell Metab 29:1003-1011.e4.

24. Zhang Y, Li X, Grailer JJ, Wang N, Wang M, Yao J, Zhong R, Gao GF, Ward PA, Tan DX, Li X. 2016. Melatonin alleviates acute lung injury through inhibiting the NLRP3 inflammasome. J Pineal Res 60:405-14.

25. Ren W, Rajendran R, Zhao Y, Tan B, Wu G, Bazer FW, Zhu G, Peng Y, Huang X, Deng J, Yin Y. 2018. Amino Acids As Mediators of Metabolic Cross Talk
between Host and Pathogen. Front Immunol 9:319.

26. He F, Wu C, Li P, Li N, Zhang D, Zhu Q, Ren W, Peng Y. 2018. Functions and Signaling Pathways of Amino Acids in Intestinal Inflammation. BioMed Research International, 2018, (2018-2-26) 2018:1-13.

27. He F, Wu C. 2018. Functions and Signaling Pathways of Amino Acids in Intestinal Inflammation. 2018:9171905.

28. Chakraborty S, Kloos B, Roetz N, Schmidt S, Eigenbrod T, Kamitani S, Kubatzky KF. 2018. Influence of Pasteurella multocida Toxin on the differentiation of dendritic cells into osteoclasts. Immunobiology 223:142-150.

29. Hildebrand D, Heeg K, Kubatzky KF. 2011. Pasteurella multocida toxin-stimulated osteoclast differentiation is B cell dependent. Infect Immun 79:220-8.

30. Ren Y, Khan FA, Pandupuspitasari NS, Zhang S. 2016. Immune Evasion Strategies of Pathogens in Macrophages: the Potential for Limiting Pathogen Transmission. Current Issues in Molecular Biology 21:21.

31. Liang N, Yang YP, Li W, Wu YY, Zhang ZW, Luo Y, Fan YM. 2018. Overexpression of NLRP3, NLRC4 and AIM2 inflammasomes and their priming-associated molecules (TLR2, TLR4, Dectin-1, Dectin-2 and NFkappaB) in Malassezia folliculitis. 61:111-118.

32. Jianghong W, Teresa FA, Alnemri ES. 2010. Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria
monocytogenes. Journal of Clinical Immunology 30:693-702.

33. Cunha TM, Talbot J, Pinto LG, Vieira SM, Souza GR, Guerrero AT, Sonego F, Verri WA, Zamboni DS, Ferreira SH. 2010. Caspase-1 is involved in the genesis of inflammatory hypernociception by contributing to peripheral IL-1β maturation. Molecular pain 6:63.

34. Hersh D, Monack DM, Smith MR, Ghori N, Falkow S, Zychlinsky A. 1999. The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc Natl Acad Sci USA 96:2396-2401.

35. Gao LN, Feng QS, Zhang XF, Wang QS, Cui YL. 2016. Tetrandrine suppresses articular inflammatory response by inhibiting pro-inflammatory factors via NF-kappaB inactivation. J Orthop Res 34:1557-68.

36. Murray PJ, Wynn TA. 2011. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 11:723-37.

37. Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T. 2013. The TSC-mTOR pathway regulates macrophage polarization. Nat Commun 4:2834.

38. Thomas W, Markus HG, Monika L. 2015. Regulation of innate immune cell function by mTOR. Nature Reviews Immunology 15:599.

39. Wang H, Zhu J, Liu Z, Lv H, Lv P, Chen F, Fu J, Hou Y, Zhao R, Xu Y. 2018. Silencing of long isoforms of nuclear factor erythroid 2 like 1 primes macrophages towards M1 polarization. Free Radical Biology & Medicine 117:37.
483 40. Kabat AM, Pearce EJ. 2017. Inflammation by way of macrophage metabolism. Science 356:488-489.
484 41. Tecchio C, Cassatella MA. 2016. Neutrophil-derived chemokines on the road to immunity. Seminars in Immunology 28:119-128.
485 42. Segal, Anthony W. 1995. Biochemistry and physiology of the neutrophil. Trends in Biochemical Sciences 20:255-256.
486 43. Eming SA, Thomas K, Davidson JM. 2007. Inflammation in wound repair: molecular and cellular mechanisms. Journal of Investigative Dermatology 127:514-525.
487 44. Hickey MJ, Kubes P. 2009. Intravascular immunity: the host-pathogen encounter in blood vessels. Nat Rev Immunol 9:364-75.
488 45. Brinkmann V, Reichard U, Goosmann C, Fautler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. 2004. Neutrophil extracellular traps kill bacteria. Science 303:1532-5.
489 46. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. 2012. Neutrophil function: from mechanisms to disease. Annu Rev Immunol 30:459-89.
490 47. Ella K, Cs’ip’ényi-K?mi R, K’eldi K. 2016. Circadian regulation of human peripheral neutrophils. Brain Behavior & Immunity 57:209-221.
491 48. Wang H, Zhang L, Yang L, Liu C, Zhang Q, Zhang L. 2017. Targeting macrophage anti-tumor activity to suppress melanoma progression. Oncotarget 8.
492 49. Greten FR, Arkan MC, Julia B, Li-Chung H, Jason G, Cornelius M, G Ktuna
SI, Michael N, Joshua F, Stephan P. 2007. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. Cell 130:918-31.

508 50. Hu Z, Murakami T, Tamura H, Reich J, K K-A, Iba T, Tabe Y, Nagaoka I. 2017. Neutrophil extracellular traps induce IL-1beta production by macrophages in combination with lipopolysaccharide. International Journal of Molecular Medicine 39:549-558.

510 51. Xia Y, Chen S, Zhu G, Huang R, Yin Y, Ren W. 2018. Betaine Inhibits Interleukin-1beta Production and Release: Potential Mechanisms. Front Immunol 9:2670.

512 52. Hara H, Seregin SS, Yang D, Fukase K, Chamaillard M, Alnemri ES, Inohara N, Chen GY, Nunez G. 2018. The NLRP6 Inflammasome Recognizes Lipoteichoic Acid and Regulates Gram-Positive Pathogen Infection. Cell 175:1651-1664 e14.

515 53. Heung LJ, Hohl TM. 2019. Inflammatory monocytes are detrimental to the host immune response during acute infection with Cryptococcus neoformans. PLoS Pathog. 15(3):e1007627. doi:10.1371/journal.ppat.1007627.

521 54. Yang Q, Ghose P, Ismail N. 2013. Neutrophils mediate immunopathology and negatively regulate protective immune responses during fatal bacterial infection-induced toxic shock. Infect Immun 81:1751-63.

524 55. Li N, Long Q, Du H, Zhang J, Pan T, Wu C, Lei G, Peng Y, Hardwidge PR. 2016. High and low-virulent bovine Pasteurella multocida capsular type A
isolates exhibit different virulence gene expression patterns in vitro and in vivo. Veterinary Microbiology 196:44-49.

56. Bo S, Huang Z, Xu X, Huang M, Wang WX, Ke C. 2015. Transcriptome analysis of the key role of GAT2 gene in the hyper-accumulation of copper in the oyster Crassostrea angulata. Sci Rep 5:17751.

57. Ren W, Yin J, Wu M, Liu G, Yang G, Xion Y, Su D, Wu L, Li T, Chen S, Duan J, Yin Y, Wu G. 2014. Serum amino acids profile and the beneficial effects of L-arginine or L-glutamine supplementation in dextran sulfate sodium colitis. PLoS One 9:e88335.

58. Thepen T, Van Rooijen N, Kraal G. 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. J Exp Med 170:499-509.

59. Brown RL, Sequeira RP, Clarke TB. 2017. The microbiota protects against respiratory infection via GM-CSF signaling. Nat Commun. 8(1):1512. doi:10.1038/s41467-017-01803-x.

60. Daley J, Thomay AM, Reichner J, Albina J. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. Journal of Leukocyte Biology 83:64-70.

61. Yu W, Zheng H, Lin W, Tajima A, Zhang Y, Zhang X, Zhang H, Wu J, Han D, Rahman NA. 2014. Estrogen promotes Leydig cell engulfment by macrophages in male infertility. Journal of Clinical Investigation 124:2709-2721.
62. Ren W, Wang P, Yan J, Liu G, Zeng B, Hussain T, Peng C, Yin J, Li T, Wei H, Zhu G, Reiter RJ, Tan B, Yin Y. 2018. Melatonin alleviates weanling stress in mice: Involvement of intestinal microbiota. J Pineal Res 64.

63. Ren W, Yin J, Xiao H, Chen S, Liu G, Tan B, Li N, Peng Y, Li T, Zeng B, Li W, Wei H, Yin Z, Wu G, Hardwidge PR, Yin Y. 2016. Intestinal Microbiota-Derived GABA Mediates Interleukin-17 Expression during Enterotoxigenic Escherichia coli Infection. Front Immunol 7:685.
Figure legends

FIG 1 Amino acid metabolism change during *P. multocida* infection. Mice were infected with *P. multocida* by intraperitoneal injection, the lung tissues were collected at 16h post-bacterial infection. (A) Alteration of glycine, serine and threonine metabolism after *P. multocida* infection (n = 3). (B) Alteration of arginine metabolism after *P. multocida* infection (n = 3). (C) The expression of genes coding the enzymes involved in the metabolism of glycine, serine and threonine metabolism, and arginine metabolism (n = 3). (D) The expression of genes coding the enzymes shown in C analyzed by qRT-PCR (n = 6). (E) The levels of amino acids in the lung tissues of mice infected by *P. multocida* (n = 6). The data were analyzed with unpaired t test. A-B: Genes in red boxes are upregulated, while in green boxes are downregulated. (D) and (E) were expressed as means ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

FIG 2 Exogenous L-serine supplementation enhances resistance to *P. multocida* infection. (A) L-serine improves the survival rate of mice (n=10). (B) L-serine markedly decreased bacterial burden in mice lungs infected by *P. multocida* at 4, 8 and 16 h post infection (n=6 or 10). (C) L-serine supplementation significantly increased the levels of L-serine in the lung tissues of mice at 8h post infection by *P. multocida* (n=10). (D)-(F) Effect of L-serine on the mRNA expression and protein abundance of IL-1β, IL-17, IFN-γ and TNF-α in the lung and serum at 4 h post infection by *P. multocida* (n=8-10). (G)-(I) Effect of L-serine on the mRNA expression and protein abundance of IL-1β, IL-17, IFN-γ and TNF-α in the lung.
and serum at 8 h post infection by *P. multocida* (n=8-10). (J) H&E staining to analyze the inflammation in the mouse lung at 16 h post infection by *P. multocida* (n=8).

(K)-(M) Effect of L-serine on the mRNA expression and protein abundance of IL-1\(\beta\), IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) in the lung and serum at 16 h post infection by *P. multocida* (n=8-10). (A), (B), (C) and (J) were pooled from three independent experiments (n = 6-10 total mice per group). (D)-(I) and (K)-(M) are representative of two independent experiments with 8-10 replicates in each time. (D-E), (G-H) and (K-L): total lung tissues were collected with half of them for RT-PCR analysis, and half for ELISA.

Data were analyzed by unpaired t-test or Mann-Whitney test, and expressed as means ±SEM. PmCQ2: *P. multocida*. *P < 0.05, **P < 0.01, ***P < 0.001.

**FIG 3** The role of L-serine on alveolar macrophage-cleared mouse model of *P. multocida* infection. Mice were infected with *P. multocida* by intraperitoneal injection, the lung tissues were collected at 16h post-bacterial infection. (A)-(D) The expression of CD68 was determined by immunohistochemistry (n=8). (E)-(F) The production of IL-1\(\beta\), IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) in the lung and serum at 16h post *P. multocida* infection (n=6). (G)-(J) The expression of Ly6G was determined by immunohistochemistry (n=8). (C), (D), (I) and (J) were determined by unpaired t-test or Mann-Whitney test, (E) and (F) were analyzed by one-way ANOVA. All data were expressed as means ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

**FIG 4** The role of L-serine on alveolar neutrophil-cleared mouse model of *P. multocida* infection. Mice were infected with *P. multocida* by intraperitoneal injection, the lung tissues were collected at 16h post-bacterial infection. (A)-(D) The expression...
of Ly6G was determined by immunohistochemistry (n=6). (E)-(F) The production of IL-1\(\beta\), IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) in the lung and serum at 16h post \(P.\) multocida infection (n=8). (G)-(J) The expression of CD68 was determined by immunohistochemistry (n=6). (C), (D), (I) and (J) were determined by unpaired t-test or Mann-Whitney test, (E) and (F) were analyzed by one-way ANOVA. All data were expressed as means ±SEM. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), ****\(P < 0.0001\).

FIG 5 Serine reduces macrophage- and neutrophil- inflammation during \(P.\) multocida infection. Mice were infected with \(P.\) multocida by intraperitoneal injection, the lung tissues were collected at 16h post-bacterial infection. (A)-(B), (E)-(F) The expression of CD68 was determined by immunohistochemistry (n=6). (C)-(D), (G)-(H) The expression of Ly6G was determined by immunohistochemistry (n=6). (I) The bacterial burden in mice lungs at 16h post \(P.\) multocida infection (n=10). (J)-(K) The production of IL-1\(\beta\), IL-17, IFN-\(\gamma\) and TNF-\(\alpha\) in the lung and serum at 16h post \(P.\) multocida infection (n=8). (E), (F), (H) and (I) were analyzed by unpaired t-test or Mann-Whitney test, (J) and (K) were determined by one-way ANOVA. All data were expressed as means ±SEM. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\), ****\(P < 0.0001\).
Figure 1
**Figure 3**

A. Empty liposome

B. Clodronate liposome

C. CD68 (ICD)

D. CD8 (ICD/core)

E. Lung

F. Serum

G. Empty liposome

H. Clodronate liposome

I. Ly6G

J. Ly6G

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Figure 4
Figure 5

A Empty liposome Control antibodies
Clodronate liposome Anti-Ly6G antibodies
CD68

B Clodronate liposome Anti-Ly6G antibodies
CD68

C Empty liposome Control antibodies
Ly6G

D Clodronate liposome Anti-Ly6G antibodies
Ly6G

E F G H I

J

Lung

IL-1β

PmcQ2

Serine

Empty liposome Control antibodies Clodronate liposome Anti-Ly6G antibodies

K

Serum

IL-17

PmcQ2

Serine

Empty liposome Control antibodies Clodronate liposome Anti-Ly6G antibodies

IFN-γ

TNF-α

ns