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Short communication

# Cofilin-1, peroxiredoxin-1, and galectin-3: Major proteins released by macrophages infected with *Corynebacterium pseudotuberculosis*

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ABSTRACT

*Corynebacterium pseudotuberculosis*, a broad host-spectrum zoonotic pathogen, causes caseous lymphadenitis (CLA) in small ruminants and is responsible for considerable economic losses in the livestock industry worldwide. Macrophages play a pivotal role in the immunopathogenesis of CLA. However, the immunoregulatory mechanisms of macrophages against *C. pseudotuberculosis* remains poorly understood. In the present study, for the first time, the partial exoproteome of murine peritoneal macrophages infected with *C. pseudotuberculosis* was profiled and the differential expression of the identified proteins was analyzed. In macrophages, infection with *C. pseudotuberculosis*, rather than with heat-killed bacteria, induced release of diverse proteins. Three unconventional proteins: cofilin-1, peroxiredoxin-1, and galectin-3 were significantly expressed and released by infected macrophages into the culture supernatant. These proteins are involved in the host inflammatory response and may be responsible for the excessive inflammation of CLA. In *C. pseudotuberculosis*–infected macrophages, the release of cofilin-1 and peroxiredoxin-1 was predominant at later stages of infection, while the release of galectin-3 was independent of time. Taken together, the present work contributes to our understanding of the functional role of macrophage response to *C. pseudotuberculosis* infection.

## 1. Introduction

*Corynebacterium pseudotuberculosis* is a facultative intracellular gram-positive bacterium that infects a broad range of hosts, including animals and humans. *C. pseudotuberculosis* causes chronic diseases such as bovine mastitis, caseous lymphadenitis (CLA) in small ruminants, ulcerative lymphangitis in horses, and necrotizing lymphangitis in humans (Trost et al., 2010; Silva et al., 2011; Barauna et al., 2017; Viana et al., 2017). CLA, a common disease in sheep and goats, shows significant epidemiological distribution and is widespread throughout the world causing great economic losses to livestock production (Connor et al., 2000). Thus, understanding the immunopathogenesis of CLA and host-*C. pseudotuberculosis* interaction is essential for developing strategies to prevent and treat this disease.

The exoproteome is a collection of proteins found in the extracellular milieu, which includes cellular secretion, other protein export mechanisms, and cell lysis. These proteins may indicate not only the physiological or pathological state of the cells in the given condition, but also the interaction between living systems and their environments (Armengaud et al., 2012). Hence, the exoproteome has an enormous potential for some novel discoveries. Difference in the exoproteome of *C. pseudotuberculosis* strains after passage in a murine (Silva et al., 2017), and that of isolates with diverse virulence have been described previously (Pacheco et al., 2011), but little is known about the released proteins in host cells. On invading the host, *C. pseudotuberculosis* is captured by phagocytic cells, such as macrophages, leading to the formation of a phagolysosome (Lopes Bastos, 2012). However, being a facultative intracellular pathogen, this bacterium survives within macrophages for more than 48 h and induces phagocytic death (Stefańska et al., 2010). In sheep and goats, macrophages are the predominant cellular composition of pyogranulomas formed in CLA and play a significant role in the immunopathogenesis of *C. pseudotuberculosis* infection (Lopes Bastos, 2012). Thus, identification of macrophage proteins in response to *C. pseudotuberculosis* infection is of importance in understanding the mechanism of bacterial pathogenesis and the associated host immune response.

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In the present study, the partial exoproteome of macrophages infected with *C. pseudotuberculosis* was profiled for the first time and the differential expression of the identified proteins between infected and uninfected macrophages was further analyzed. Subsequently, the possible relevance of the release and the biological functions of these proteins are discussed.

## 2. Materials and methods

### 2.1. Mice

Seven to eight-week-old female C57BL/6 mice used in the present study were purchased from the Chongqing Academy of Chinese Materia Medical (Chongqing, China). Mice were housed in clean cages at a temperature of 24 ± 2 °C and relative humidity of 55 ± 15%. All animal experiments and procedures were approved by the Laboratory Animal Ethical Commission of Southwest University (Chongqing, China).

### 2.2. Bacteria

*Corynebacterium pseudotuberculosis* (ATCC 19410) was purchased from Guangdong Culture Collection Centre (Guangzhou, China). The bacteria were cultured in nutrient broth (AOBOX, China) supplemented with 10% horse serum (Biological Industries, Israel) at 37 °C for 24 h.

### 2.3. Isolation of murine peritoneal macrophages

Murine peritoneal macrophages were isolated as described previously (Feng et al., 2018) with minor modifications. Briefly, 2 mL of 4% sterile thioglycolate medium (Eiken Chemical Co., Ltd, Japan) was intraperitoneally (i.p) injected into mice 4 days prior to cell harvest. Peritoneal exudate cells (PECs) were harvested by peritoneal lavage after euthanasia. Briefly, 5 mL of pre-cooled Roswell Park Memorial Institute (RPMI) 1640 medium (Biological Industries, Israel) was injected into each mouse and the collected peritoneal fluid was centrifuged at 1800 rpm for 5 min at 4 °C. Cell pellets were washed and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), plated at a density of $10^7$ cells/well in 6-well plates, and incubated for 2 h at 37 °C in a humidified incubator supplied with 5% $CO_2$. The non-adherent cells were removed by gently washing twice with RPMI 1640 medium supplemented with 10% FBS. More than 95% of the adherent cells were F4/80+ macrophages, as determined by immunofluorescence using F4/80 monoclonal antibody (eBioscience, USA).

### 2.4. Stimulation of peritoneal macrophages by C. pseudotuberculosis infection

Peritoneal macrophages were infected with *C. pseudotuberculosis* at a multiplicity of infection (MOI) of 60 or inoculated with same amount of heat-killed bacteria (*C. pseudotuberculosis* were killed by placing them in a boiling water bath for 5 min) and left uninfected as controls. The cells were incubated for 1 h in RPMI 1640 medium supplemented with 10% FBS at 37 °C, washed twice with PBS, cultured in Opti-minimal essential medium (MEM) (Gibco, USA) containing 100 μg/mL gentamicin to kill bacteria outside of the cells, and additionally incubated for the corresponding time (the total time for infection was 1 h plus the corresponding time), following which the culture medium was collected and centrifuged at 2000 × *g* for 15 min. Supernatants were transferred to new centrifuge tubes for further analysis.

### 2.5. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All culture supernatants were concentrated 10-fold using the Pierce protein concentrators, 3 K molecular weight cutoff (MWCO) (Thermo Scientific, United Kingdom) as per the manufacturer's instructions. The protein concentrations were determined using a BCA protein assay kit (Beyotime, China). The concentrated protein samples were mixed with the SDS-PAGE sample loading buffer (Beyotime, China) and incubated at 100 °C for 5 min in a water bath. Twenty microliters of the prepared samples and 5 μL of the PageRuler™ prestained protein ladder (Thermo Scientific, United Kingdom) were separated on 4–20% SDS-PAGE gel using the Mini-PROTEAN® 3 electrophoresis system (Bio-Rad, USA). After electrophoresis, separated proteins were visualized by staining the gel using the Coomassie blue staining kit (Beyotime, China) as per the manufacturer's protocol.

### 2.6. Nano-liquid chromatography tandem-mass spectrometry analyses

Gel slices containing the desired protein bands were incised and digested with sequencing-grade modified trypsin (Promega, USA). Proteins were identified using the Nano-LC-ESI MS/MS system performed by Suzhou ProtTech, Inc (Suzhou, China). Mass spectrometric data were searched against the UniProt protein database with ProtTech's ProtQuest software suite (Philadelphia, PA, USA). Identified proteins from each sample are listed along with the calculated relative protein abundance.

### 2.7. Functional annotation

The identified proteins with the highest confidence limits (99.0%) were annotated with level-2 Gene ontology (GO) terms and classified into functional categories by GO annotation using the online annotation clustering tool The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (http://david.abcc.ncifcrf.gov/).

### 2.8. Quantitative polymerase chain reaction (qPCR) analyses

Total RNA was extracted from peritoneal macrophages (cultured in Opti-MEM at a density of $2 \times 10^6$ cells/well in 12-well plates), with or without *C. pseudotuberculosis* infection, using RNAiso plus (TaKaRa, China) according to the manufacturer's protocol. Complementary DNA was synthesized using a PrimeScript™ RT reagent kit (TaKaRa, China). qPCR was performed using TB Green gene expression assays (TaKaRa, China) on a CFX96 Real-time PCR detection system (Bio-Rad, USA). Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method with β-actin as the endogenous housekeeping gene. Primer pairs specific for the coding genes of proteins selected from mass spectrometry results were synthesized by Bioligo Biotechnology Co., Ltd. (Shanghai, China). Details of the primer sequences used in the present study are listed in Table 1.

### 2.9. Western blot analyses

Lysate of peritoneal macrophages ($5 \times 10^6$ cells/well), with or without *C. pseudotuberculosis* infection, was prepared using RIPA buffer (Invitrogen, USA) as per the manufacturer's protocol. Lysates were then mixed with the loading buffer. The concentrated culture supernatants described above and cell lysates were separated on 15% SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by electroblotting. The membranes were blocked with 5% non-fat dry milk for 1 h and incubated overnight at 4 °C with the

**Table 1**
Primers for detecting mRNA expression of target proteins.

| Proteins | Genes | Primer sequences (5' - 3') | |
|---|---|---|---|
| Profilin-1 | *Pfn1* | Forward | AACGCCTACATCGACAGCC |
| | | Reverse | CGTAATGCTAACGAAGGTCTTCC |
| Cofilin-1 | *Cfl1* | Forward | ATGACATGAAGGTTCGCAAGT |
| | | Reverse | GACAAAAGTGGTGTAGGGGTC |
| Peroxiredoxin-1 | *Prdx1* | Forward | AATGCAAAAATTGGGTATCCTGC |
| | | Reverse | CGTGGGACACACAAAAGTAAAGT |
| Galectin-3 | *Lgals3* | Forward | TGCTGGTTCCAGGGACTCAA |
| | | Reverse | CCACCGGCCTCTGTAGAAGA |
| Cathepsin B | *Ctsb* | Forward | TCCTTGATCCTTCTTTCTTGCC |
| | | Reverse | ACAGTGCCACACAGCTTCTTC |
| Arginase-1 | *Arg1* | Forward | CTCCAAGCCAAAGTCCTTAGAG |
| | | Reverse | AGGAGCTGTCATTAGGGACATC |
| Cathepsin D | *Ctsd* | Forward | GCTTCCGGTCTTTGACAACCT |
| | | Reverse | CACCAAGCATTAGTTCTCCTCC |
| Vimentin | *Vim* | Forward | CGTCCACACGCACCTACAG |
| | | Reverse | GGGGGATGAGGAATAGAGGCT |
| Beta-actin | *β-actin* | Forward | CTAAGGCCAACCGTGAAAAG |
| | | Reverse | ACCAGAGGCATACAGGGACA |

following antibodies: anti-cofilin-1 (Sangon Biotech, China), anti-peroxiredoxin-1 (Sangon Biotech, China), and anti-galectin-3 (Boster Biological Technology, China). The membranes were washed three times using Tris buffered saline with Tween 20 (TBST), incubated with HRP-conjugated secondary antibodies, and visualized using an ECL detection reagent (Beyotime, China) as per the manufacturer's instructions.

### 2.10. Statistical analysis

The results of qPCR were reported as means ± standard error. Student's *t*-test was performed using SPSS Statistics 24.0 software. *P*-values of $< 0.05$ were considered significant ($^{*}P < 0.05$ and $^{**}P < 0.01$).

## 3. Results

### 3.1. Release of diverse proteins by macrophages stimulated with C. Pseudotuberculosis

The infection of *C. pseudotuberculosis*, rather than heat-killed bacteria, induced large amounts of protein release from macrophages compared to uninfected macrophages (Fig. 1A). In total, 180 extracellular proteins were identified in culture supernatants and three of them were derived from *C. pseudotuberculosis* (Supplementary Table). Identified proteins from each band with the highest confidence limit and highest relative abundance are listed in Fig. 1B. In order to deeply analyze the identified proteins and the protein profiles, we have functionally classified the released proteins from macrophages stimulated with *C. pseudotuberculosis* using Gene ontology (GO) annotation. To ensure the quality of functional analysis, 76 proteins with the highest confidence limits (99.0%) were selected to be annotated with level-2 GO terms and were categorized into three major GO categories (Fig. 1C). The most enriched term in the molecule function (MF) category was protein binding (56 proteins, GO:0005515). For cellular component (CC) category, a high percentage of proteins were related to the extracellular exosome term (69 proteins, GO:0070062). In the biological process (BP) category, a large number of proteins were related to single-organism process (60 proteins, GO:0044763).

### 3.2. Enhanced mRNA expression of cofilin-1, peroxiredoxin-1, and galectin-3 in macrophages infected with C. Pseudotuberculosis

The mRNA expression of eight proteins of interest was further evaluated by qPCR analyses (Fig. 2). The results indicated significantly enhanced mRNA expression levels of cofilin-1 (*Cfl1*), peroxiredoxin-1 (*Prdx1*), and galectin-3 (*Lgals3*) in macrophages 18 h after *C. pseudotuberculosis* infection. *Lgals3* expression was upregulated at all detected time points after *C. pseudotuberculosis* infection, while that of *Cfl1* and *Prdx1* was significantly reduced at 6 h and 12 h post infection. Interestingly, the mRNA expression of the other genes was suppressed in *C. pseudotuberculosis*-infected macrophages. The mRNA expression of cathepsin B (*Ctsb*) and cathepsin D (*Ctsd*) was significantly reduced at all detected time points after *C. pseudotuberculosis* exposure. Arginase-1 (*Arg1*) and vimentin (*Vim*) were only significantly suppressed after 12 h in *C. pseudotuberculosis*-infected macrophages, while the mRNA expression of profilin-1 (*Pfn1*) was significantly downregulated at both 6 h and 12 h post infection.

### 3.3. Alterations in protein expression of cofilin-1, peroxiredoxin-1, and galectin-3 in macrophages infected with C. Pseudotuberculosis

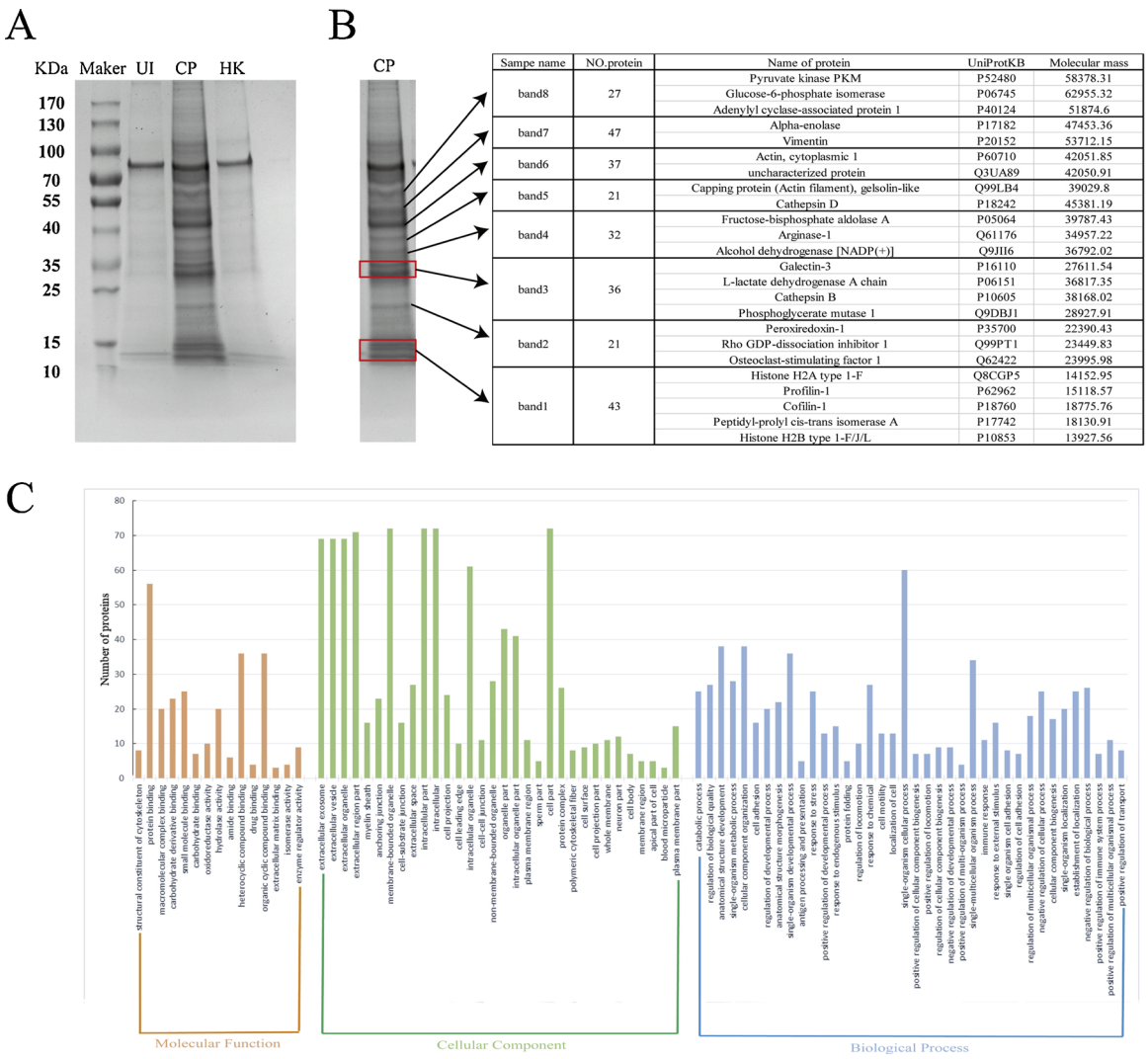
To further validate the molecules with enhanced mRNA expression, alterations in protein expression of cofilin-1, peroxiredoxin-1, and galectin-3 in macrophages infected with *C. pseudotuberculosis* were analyzed by western blot. Cofilin-1 and peroxiredoxin-1 were mainly released into the media by macrophages at 24 h post-infection indicating late stage expression of these proteins. Enhanced release of galectin-3 was also detected in the supernatant of macrophages after *C. pseudotuberculosis* infection; however, there was no obvious difference between all detected time points (12 h-, 18 h-, and 24 h-post infection). Progressively enhanced expression of peroxiredoxin-1 but not galectin-3 and cofilin-1 was detected in the macrophage cell lysate infected with *C. pseudotuberculosis* (Fig. 3).

## 4. Discussion

Screening and identification of proteins released by macrophages infected with *C. pseudotuberculosis* are of prime importance in understanding the mechanism of host immune response to this pathogen. In the present study, the partial exoproteome of murine peritoneal macrophages infected with *C. pseudotuberculosis* was profiled. Eight proteins identified by Nano-LC-ESI-MS/MS were further studied to analyze the pattern of mRNA expression.

The mRNA expression levels of cofilin-1, peroxiredoxin-1, and galectin-3 were significantly enhanced in macrophages infected with *C. pseudotuberculosis* at 18 h. However, whether the enhanced mRNA expression contributes to the host immune response against bacteria and the underlying regulatory mechanisms need to be fully elucidated. Interestingly, we found that the expression of certain genes was significantly suppressed at some detected time points after *C. pseudotuberculosis* infection. We speculated that this suppression of gene expression in the host may be related to the requirement of specific environment for the replication of *C. pseudotuberculosis* in macrophages, which is worthy of further research. In addition to analyzing the expression level of the transcripts to predict protein levels, enhanced expression of the identified proteins was validated by western blot analyses, which confirmed significant release of cofilin-1, peroxiredoxin-1, and galectin-3 into the culture media by macrophages infected with *C. pseudotuberculosis*.

Cofilin-1 is a cytoskeletal protein essential for several cellular processes such as cytokinesis and endocytosis (Hotulainen et al., 2005;




| Sampe name | NO.protein | Name of protein | UniProtKB | Molecular mass |
|---|---|---|---|---|
| band8 | 27 | Pyruvate kinase PKM | P52480 | 58378.31 |
| | | Glucose-6-phosphate isomerase | P06745 | 62955.32 |
| | | Adenylyl cyclase-associated protein 1 | P40124 | 51874.6 |
| band7 | 47 | Alpha-enolase | P17182 | 47453.36 |
| | | Vimentin | P20152 | 53712.15 |
| band6 | 37 | Actin, cytoplasmic 1 | P60710 | 42051.85 |
| | | uncharacterized protein | Q3UA89 | 42050.91 |
| band5 | 21 | Capping protein (Actin filament), gelsolin-like | Q99LB4 | 39029.8 |
| | | Cathepsin D | P18242 | 45381.19 |
| band4 | 32 | Fructose-bisphosphate aldolase A | P05064 | 39787.43 |
| | | Arginase-1 | Q61176 | 34957.22 |
| | | Alcohol dehydrogenase [NADP(+)] | Q9JI6 | 36792.02 |
| band3 | 36 | Galectin-3 | P16110 | 27611.54 |
| | | L-lactate dehydrogenase A chain | P06151 | 36817.35 |
| | | Cathepsin B | P10605 | 38168.02 |
| | | Phosphoglycerate mutase 1 | Q9DBJ1 | 28927.91 |
| band2 | 21 | Peroxiredoxin-1 | P35700 | 22390.43 |
| | | Rho GDP-dissociation inhibitor 1 | Q99PT1 | 23449.83 |
| | | Osteoclast-stimulating factor 1 | Q62422 | 23995.98 |
| band1 | 43 | Histone H2A type 1-F | Q8CGP5 | 14152.95 |
| | | Profilin-1 | P62962 | 15118.57 |
| | | Cofilin-1 | P18760 | 18775.76 |
| | | Peptidyl-prolyl cis-trans isomerase A | P17742 | 18130.91 |
| | | Histone H2B type 1-F/J/L | P10853 | 13927.56 |

**Fig. 1.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Nano-LC-ESI-MS/MS based profiling and identification of extracellular proteins released by macrophages stimulated with Corynebacterium pseudotuberculosis. A. Macrophages ($1 \times 10^7$) were stimulated with *C. pseudotuberculosis* (multiplicity of infection (MOI) = 60) or incubated with heat-killed *C. pseudotuberculosis* for 1 h. The cells were washed twice with phosphate buffered saline (PBS) and cultured in Opti- minimal essential medium (MEM) containing 100 μg/mL gentamicin for 17 h (the total time for infection was 18 h). After incubation, culture supernatants were collected, concentrated, and separated on 4–20% SDS-PAGE gel and the separated proteins were visualized by Coomassie blue staining. B. Differential bands from the CP group were incised and subsequently identified by Nano-LC-ESI-MS/MS. Proteins identified with the highest confidence limits and highest relative abundance corresponding to each band are listed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article). **C.** The identified extracellular proteins with highest confidence limits(99.0%)were annotated by Gene ontology (GO) annotation (2nd level GO terms). The proteins were classified into molecular function (MF), cellular component (CC), and biological process (BP) categories. CP: *C. pseudotuberculosis*; HK: heat-killed *C. pseudotuberculosis*; UI: uninfected.

Okreglak and Drubin, 2007). Cofilin-1 released in an exosome in the extracellular environment might influence the capacity of macrophages to migrate to the site of infection/inflammation, and carry molecules such as antigens of bacteria and/or components from infected macrophages to elicit immune responses in resting cells (Wang et al., 2014). In addition, cofilin-1 is a key component in regulating the activation of the NLRP3 inflammasome, suggesting its involvement in the host immune response (Park et al., 2015). Further studies related to the biological function of cofilin-1 in response to *C. pseudotuberculosis* infection and the related pathogenesis is of research interest.

Peroxiredoxin-1 is a ubiquitous antioxidant enzyme that protects the cells against oxidative stress and helps in maintaining redox homeostasis (Immenschuh and Baumgart-Vogt, 2005). Although it was originally described as an endogenous protein, previous studies have reported caspase-1-dependent secretion of peroxiredoxin-1 through a nonclassical secretory pathway (Geiben-Lynn et al., 2003; Keller et al., 2008). Extracellular peroxiredoxin-1 is associated with multiple innate immune mechanisms such as enhancing natural killer (NK) cell activity, regulating inflammatory reactions (Shau et al., 1993; Jung et al., 2001), and stimulating the release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β through the nuclear factor kappa B (NF-κB) signaling pathway (Riddell et al., 2010; Liu et al., 2018). We had previously found that in macrophages, *C. pseudotuberculosis* infection could induce the secretion of IL-1β and TNF-α, and expression of

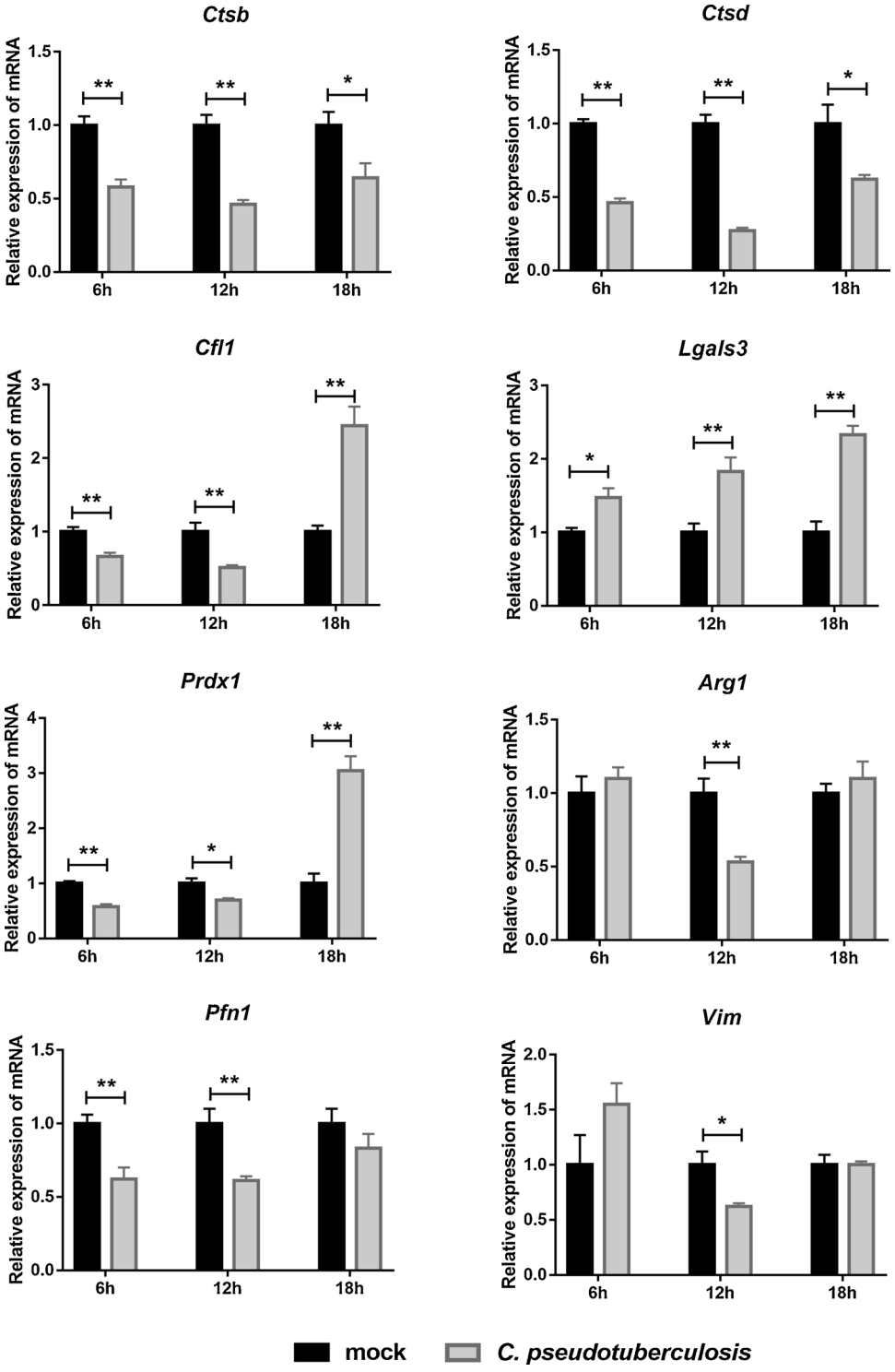



**Fig. 2.** Quantitative polymerase chain reaction (qPCR) analyses of eight selected proteins released by macrophages stimulated with *Corynebacterium pseudotuberculosis*. Macrophages ($2 \times 10^6$) were infected with *C. pseudotuberculosis* at a multiplicity of infection (MOI) of 60 for 1 h, washed twice with phosphate buffered saline (PBS), and cultured in Opti-minimal essential medium (MEM) containing 100 μg/mL gentamicin, for 5, 11, and 17 h (the total time for infection was 6, 12, and 18 h). Uninfected macrophages served as controls. mRNA expression was analyzed by qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method and the results are presented as the means ± standard error (n = 3). *, $P < 0.05$; **, $P < 0.01$.

NLRP3 (data not shown). Further studies are required to confirm the involvement of cofilin-1 and peroxiredoxin-1 in the secretion of IL-1β and TNF-α. Since cofilin-1 and peroxiredoxin-1 are released by macrophages at a later stage of *C. pseudotuberculosis* infection, their role in causing excessive inflammation needs to be elucidated.

Galectin-3 is a β galactoside binding lectin and is implicated in fine-tuning innate immune responses (Ferraz et al., 2008). Secreted galectin-3 can act as a pattern-recognition receptor (PRR) and as an immunomodulator in the extracellular medium, recognizing microbial structures and promoting the assembly of immune cells to the infected site (Sato et al., 2009). CLA was characterized by the maturation and persistence of pyogranuloma, which has a complex cellular composition

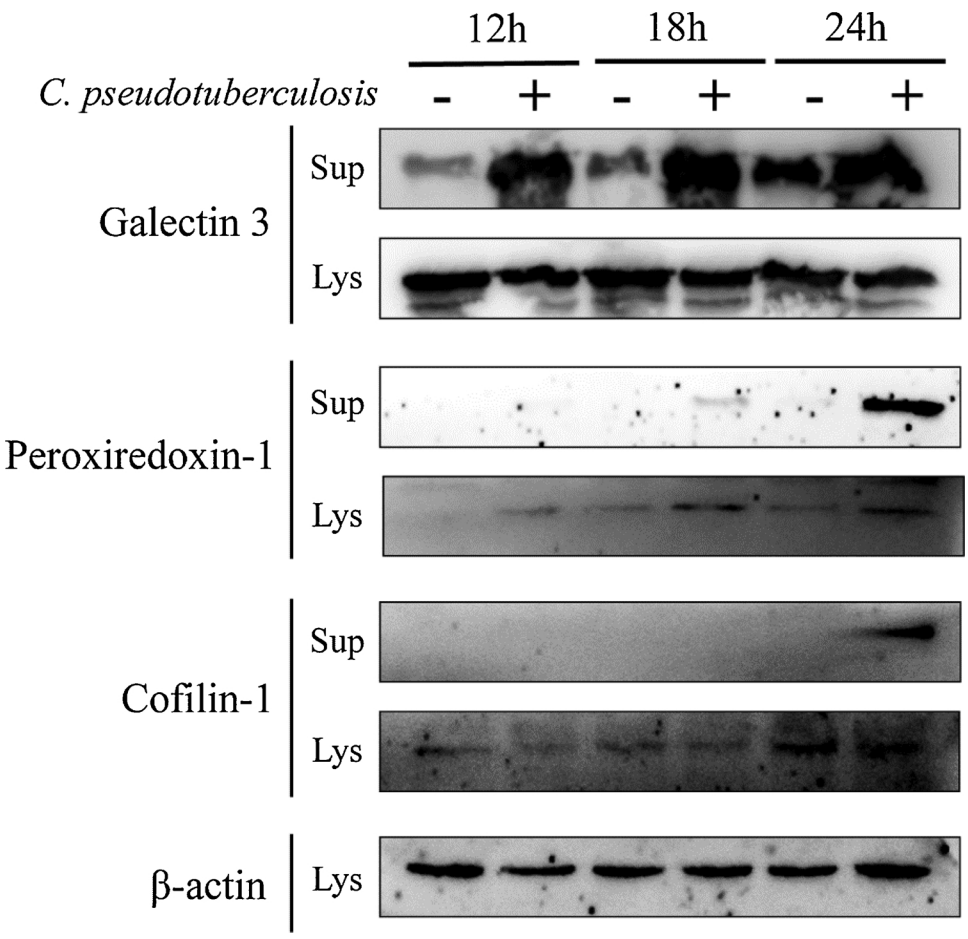



**Fig. 3.** Altered protein expression of cofilin-1, peroxiredoxin-1, and galectin-3 in culture supernatants and cell lysates of macrophages infected with *Corynebacterium pseudotuberculosis*. Macrophages ($5 \times 10^6$) were infected with *C. pseudotuberculosis* at a multiplicity of infection (MOI) of 60 for 1 h, washed twice with phosphate buffered saline (PBS), and cultured in Opti-minimal essential medium (MEM) containing 100 μg/mL gentamicin, for 11, 17, and 23 h (the total time for infection was 12, 18, and 24 h). Expression levels of cofilin-1, peroxiredoxin-1, and galectin-3 were detected by western blotting. The experiments were repeated three times with similar results. Sup: supernatants; Lys: cell lysate.

(Lopes Bastos, 2012). Thus, we speculate that Galectin-3 is involved in the development of pyogranuloma in CLA. In addition, galectin-3 plays a prominent role in inflammation upon infection-initiated tissue damage (Díaz-Alvarez and Ortega, 2017). In the present study, enhanced release of galectin-3 into the culture medium but not in the cell lysate was seen in macrophages infected with *C. pseudotuberculosis*, suggesting a vital role of galectin-3 in the extracellular environment in response to *C. pseudotuberculosis* infection. However, the functional mechanism still needs to be elucidated.

In conclusion, the present study analyzed the partial exoproteome of macrophages infected with *C. pseudotuberculosis* and identified enhanced release of cofilin-1, peroxiredoxin-1, and galectin-3 in the culture supernatants. However, whether the accumulation of these proteins is related to bacterial replication, and their function in the extracellular milieu as well as the secretory pathways remain to be fully elucidated. This study provides a novel insight into the extracellular protein profile of macrophages involved in functional response to *C. pseudotuberculosis* infection.

## Declaration of Competing Interest

None of the authors have any conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108461.

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