Microbial Pathogenesis 119 (2018) 225–232

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

# Effects of *Pasteurella multocida* lipopolysaccharides on bovine leukocytes

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ARTICLE INFO

*Keywords:*
Apoptosis
Bovine leukocytes
Lipopolysaccharide
Lymphocytes
Monocytes
Macrophages
Necrosis
Neutrophils
*Pasteurella multocida*
Pyroptosis

ABSTRACT

Lipopolysaccharide (LPS) is a major virulence factor of Gram-negative bacteria playing a major role in stimulating protective immune response in mammalian host. However, in many gram-negative bacterial infections, LPS also elicits immunopathology by inducing excessive inflammatory changes. *P. multocida* (Pm), a gram-negative bacterium, causes acute lung inflammation and fatal septicemic disease in animals. However, the effects of Pm LPS on host cells are little known. In this study, LPS isolated from three different serotypes (B:2, A:1 and A:3) of Pm were individually tested *in vitro* to assess the response of bovine leukocytes. Pm LPS induced cell proliferation and cell death of leukocytes, in a dose- and time-dependent manner. In these cells, mitochondrial dysfunction and caspase activation mediate cell death.

## 1. Introduction

*Pasteurella multocida* is an opportunistic pathogen known for its multi-species commensalism and causing a variety of diseases in mammals and birds. The isolates of *P. multocida* (Pm) are normally found in the upper respiratory tract of healthy animals, but it is elusive how these isolates cause acute disease at times. In animals, Pm causes fatal septicemic disease, which is characterized by acute inflammation in lungs and other vital organs [1–5]. The cell wall components of Pm including capsule, LPS, surface fimbria, and outer membrane proteins are identified as important virulence factors [6–8]. Previous studies have shown that in vivo injection of Pm LPS elicited endotoxemic response in animals and birds [9–13] However, little is known on the effect of Pm LPS interaction with bovine leukocytes.

Many studies have characterized the structure of *P. multocida* LPS inner and outer core regions for a large number of strains, including the 16 Heddleston type strains (reviewed in 14). Interestingly, most *P. multocida* strains produce two inner core structures (glycoform A and glycoform B) and these inner core oligosaccharide structures are identical across all the *P. multocida* strains [7,8]. However, the outer core structures are different among various serotypes. Excluding serovars 2 and 5, other Heddleston type strains express structurally distinct outer core regions, which also lack repeating polymeric O-antigen in all cases [14]. Overall, *P. multocida* LPS is structurally diverse with greater distinction in the outer core structure due to mutations within individual biosynthesis/transferase genes in the LPS outer core locus [7]. On the other hand, there is limited information on primary structure of the lipid A molecule produced by *P. multocida* [14].

In Gram-negative bacterial infections, LPS (endotoxin) is a major virulence factor that plays a key role in stimulating protective immunity to clear infections. However, an uncontrolled inflammatory response to LPS results in excessive inflammation and tissue damage [15–17]. These pathological changes are mediated by LPS-stimulated cytokines/chemokines [16,18]. While these cytokines/chemokines elicit immune responses, bacterial endotoxins can also activate cell death and subsequent tissue damage [19–23]. An elegant study using electron microscopy has revealed that binding and internalization of LPS across plasma membrane of human monocytes caused disruption of organelles and nuclear membranes [24]. It has also been reported that LPS induces cell death through several mechanisms [23–29]. However, many scientific experts of endotoxin biology may still believe that LPS-induced cell death could be an experimental artifact. Nevertheless, many reports provide evidence for LPS-induced cell death [24–28]. Interestingly, recent studies have shown a novel mechanism in that intracellular localization and recognition of LPS by inflammasome components, independent of TLR-4 signaling, activated caspase-1/11-dependent cell death, [30–32]. It was previously reported that *Mannheimia heamolytica* LPS and LKT caused cell death in bovine leukocytes and endothelial cells [19,22,28]. We have reported that Pm infection induced cell death in circulating and lung infiltrating leukocytes [4], and it caused

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https://doi.org/10.1016/j.micpath.2018.04.030
Received 11 January 2018; Received in revised form 30 March 2018; Accepted 16 April 2018
Available online 17 April 2018

extensive necrosis of lung tissues [5]. However, the mechanisms of Pm LPS-induced cell death and tissue pathology are unknown.

In this study, we investigated the effects of Pm LPS interaction with bovine leukocytes, specifically focusing on ultrastructural changes and cell death analyses, and determined that LPS caused mitochondrial dysfunction leading to cell death in leukocytes exposed to LPS.

## 2. Materials and methods

### 2.1. Pasteurella multocida and LPS purification

Isolates of *P. multocida* serotypes B:2 (P52), A:1 (P120) and A:3 (P30) used in this study were obtained from Bacteriology Divison of the Institute. These isolates were cultured in brain heart infusion broth as described [4] and LPS was isolated by phenol-water extraction method [33]. The crude LPS preparations were treated with proteinase K (10 μg/ml), DNase (2 μg/ml) and RNase (1 μg/ml) to remove proteins and nucleic acid contaminants. The endotoxin levels (EU/mg of dry weight) in LPS preparations were quantified as $3.2 \times 10^5$ (Pm B:2), $2.7 \times 10^5$ (Pm A:1) and $3 \times 10^5$ (Pm A:3) by *Limulus* Ameobocyte Lysate assay (Associates of Cape Cod, MA, USA). The protein level was found to be $< 0.01$ μg/ml in LPS preparations.

### 2.2. Peripheral blood leukocyte isolation and stimulation with LPS

Peripheral blood leukocytes were isolated from healthy cattle blood by density-gradient centrifugation procedure as described [34]. Briefly, fresh blood was layered over Histopaque-1077 (Sigma, MO, USA), centrifuged and peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat layer. The total cell counts were determined and cell numbers were adjusted to $2 \times 10^6$ cells/ml. PBMC were cultured in RPMI medium containing 10% FBS. The subsets of mononuclear leukocytes (e.g., lymphocytes, monocytes and macrophages) were fractionated on the basis of cell adherence to culture plates. After 3 h incubation of PBMC, the non-adherent cells (lymphocytes) were collected and cultured separately in 24-well plates ($1 \times 10^6$ cells/well). The adherent monocytes were washed twice and cultured separately. Some of the adherent cells were cultured for 7–8 days to obtain monocyte-derived macrophages [34]. Neutrophils were isolated from the bottom granulocyte-rich layer (just above the erythrocytes) of the centrifuged blood. The granulocyte-rich layer was mixed with equal volume of the dextran-saline, left undisturbed for 15 min and neutrophils were isolated. The neutrophils were cultured in RPMI. The subsets of bovine leukocytes were confirmed by the nuclear morphology on Giemsa staining. In addition, macrophages were further confirmed by α-napthyl acetate esterase staining [34]. PBMC or leukocyte subsets were treated with Pm LPS (0.1, 1 and 10 μg/ml) and kinetics of cellular responses were studied at different time-points. Cell death analyses were done between 3 and 12 h (for lymphocytes and neutrophils) or 12–48 h (for monocytes and macrophages) post-LPS treatment.

### 2.3. Real-time qPCR for cytokine and apoptotic gene expressions

The expression pattern of cytokine and apoptotic genes were performed by qPCR using bovine-specific primers (Supplemental Table 1) and expression levels (as fold change) were calculated as described [34].

### 2.4. Cell proliferation and cytotoxicity assays

PBMC proliferation was analyzed by tetrazolium salt (MTT) reduction assay, in which tetrazolium salt (Sigma) was reduced by enzymes of viable cells into formozan that measures the rate of cell proliferation. The per cent cytotoxicity was quantified by using CytoTOX-ONE™ membrane integrity assay (Promega) that measures the amount of lactate dehydrogenase (LDH) release into culture medium [34]. Cells treated with SDS (0.1%) were considered as positive control for maximum release of LDH.

### 2.5. Detection of apoptosis and necrosis

In LPS-treated leukocytes, live and dead cells were identified by nuclear morphology and cellular permeability to DNA intercalating dyes (*e.g.,* AO/EB, DAPI/PI or annexin V/PI). Briefly, Pm LPS-treated cells were harvested and stained with DNA dyes and examined under fluorescence microscopy. The frequencies of apoptotic and necrotic cells were calculated as described [34].

### 2.6. Transmission electron microscopy (TEM)

Ultrastructural changes of live and dead cells were studied by transmission electron microcopy as described [33].

### 2.7. Caspase-3 assay

Caspase-3 activity was measured using CaspACE™ colorimetric assay (Promega) as described [34]. In some experiments, pan-caspase (Z-VAD-fmk) and caspase-3 (Z-DEVD-fmk) inhibitors (Promega) were used to assess the role of caspases in LPS-induced apoptosis. Staurosporine (Sigma) at 10 μM final concentration was used as a positive control for apoptosis. Cells were treated with pan-caspase (40 μM) or caspase-3 (50 μM) inhibitor for 30 min prior to LPS stimulation, as described [34].

### 2.8. Nitric oxide (NO) quantification

NO produced by macrophages was measured with Griess reagent (Sigma). In some experiments, NO inhibitor (L-NAME) at 100 μM/ml concentration was used 30 min prior to LPS treatment, to assess the role of NO in apoptosis [34].

### 2.9. Mitochondrial membrane potential (ΔΨm) assay

The change in ΔΨm in leukocytes was measured by using $DiOC_6$ [3] as described previously [34]. The role of mitochondrial permeability transition (MPT) due to change of ΔΨm for apoptosis was tested by using MPT inhibitor, cyclosporine A (CsA), at 1 μM/ml final concentration 30 min prior to LPS treatment [34]. As a positive control, CCCP (carbonyl cyanide 3-chlorophenyl-hydrazone), which disrupt ΔΨm, was used (50 μM conc.) to check the effect of MPT inhibitor.

### 2.10. Statistical analysis

Results are expressed as mean ± SD. Data comparisons between groups were performed by an ANOVA with Tukey's post-test or Student's *t* test in GraphPad Prism 4 software (GraphPad, San Diego, CA). A $p < 0.05$ value was considered statistically significant.

## 3. Results

### 3.1. Pm LPS induces proliferation and cytokine gene expressions in bovine PBMC

*In vitro* exposure of PBMC with Pm LPS induced cell proliferation and cytotoxicity, in a dose-and time-dependent manner (Supplemental Table 2). The MTT assay, as a measure of cell proliferation, showed an increased cell proliferation in PBMC (Fig. 1A). Interestingly, Pm LPS at higher concentration (10 μg/ml) induced cell death in PBMC at 12–24 h (Fig. 1B). The mRNA profiling of cytokine genes, as a measure of cell activation, showed a higher expression of TNF-α, IL-1β, IL-6, IL-8, IL-12 and INF-γ genes, while lower expression of IL-2, IL-4, IL-10 and TGF-β

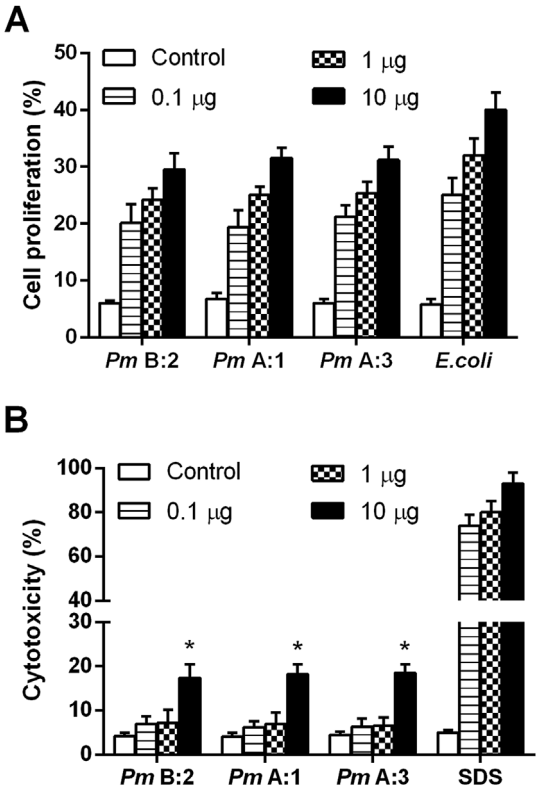


**Fig. 1.** (**A**) Per cent cell proliferation in bovine PBMC calculated by MTT assay at 24 h post-treatment with Pm LPS (0.1–10 μg/ml). (**B**) Per cent cytotoxicity in bovine PBMC calculated by LDH assay at 24 h post-treatment with Pm LPS (0.1–10 μg/ml). Data represent mean ± SD from three independent experiments (Student' *t*-test, *p < 0.05, **p < 0.01).

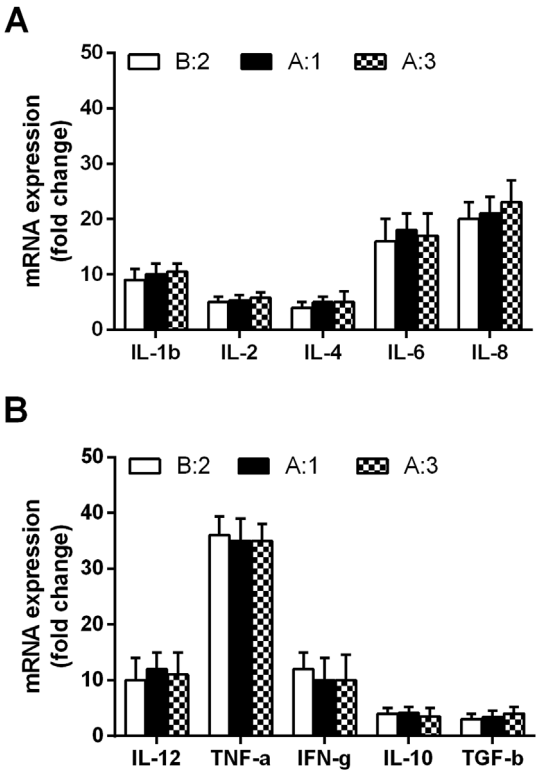


**Fig. 2.** (**A-B**) Cytokine gene expression in bovine PBMC calculated by qPCR at 12 h post-treatment with Pm LPS (1 μg/ml). Data represent mean ± SD from three independent experiments.

genes (Fig. 2A–B).

### 3.2. Pm LPS induces cell death in bovine leukocytes

To investigate the kinetics and mechanisms of cell death induced by Pm LPS, bovine lymphocytes, neutrophils, monocytes and macrophages were isolated, cultured separately and treated with Pm LPS (10 μg/ml). The microscopic analyses of cell death observed in lymphocytes are shown in Fig. 3. On phase-contrast, control cells showed normal cell morphology with high phase-contrast refractivity, while staurosporine-treated or LPS-treated lymphocytes showed apoptotic changes like membrane blebs and cellular fragmentation. On AO/EB staining, apoptotic cells were identified with condensed (bright green) or fragmented nuclei, while necrotic cells as round (red) nuclei. On DAPI staining, live cells showed normal nuclei (blue), while apoptotic cells showed condensed or fragmented nuclei (deep blue). On annexin V/PI staining, early apoptotic (green) apoptotic (reddish-green) and necrotic (red) cells were also identified clearly (Fig. 3).

Pm LPS at higher concentration (10 μg/ml) induced apoptosis in lymphocytes as early as 3 h and numbers of apoptotic cells were increased at 12 h (Fig. 4A). Bovine neutrophils exposed to Pm LPS showed a delay in apoptosis at 3 and 6 h, while higher ($p < 0.05$) apoptotic cells were observed at 12 h (Fig. 4B). In contrast, Pm LPS induced apoptosis in monocytes and macrophages at longer (> 24 h) exposure time (Fig. 4C–D). In addition, significant numbers of necrotic cells were observed in neutrophils and macrophages at later time-points following LPS exposure (data not shown).

### 3.3. Electron microscopy of Pm LPS-induced cell death in bovine leukocytes

On TEM analysis, untreated control lymphocyte showed normal ultrastructural features such as round or slightly oval shaped cell with little cytoplasm and normal nucleus (Fig. 5A). Normal distributions of heterochromatin and euchromatin were seen. In contrast, LPS-treated lymphocytes showed many ultrastructural changes consistent with apoptotic features like chromatin condensation and margination, nuclear and cellular contraction, nuclear fragmentation, and apoptotic body formation (Fig. 5B–F). LPS-treated lymphocytes showed alterations in nuclear shape such as involution or contraction. Nuclear chromatin condensation and margination to nuclear membrane formed the typical features of apoptosis in lymphocytes (Fig. 5B). Chromatin condensation in the form of horse-shoe or half-moon shape was frequently observed in LPS-treated lymphocytes (Fig. 5B–C). Round apoptotic micronuclei filled with condensed chromatin were also seen (Fig. 5C, arrow). The fragmented nuclear structures were often connected by a thin line of nuclear membrane (Fig. 5D). Two types of chromatin margination (complete or irregular) were observed in LPS-treated lymphocytes. The entire chromatin structures were moved towards the periphery of a cell uniformly in complete type (Fig. 5D), while a large amount of chromatin was moved to one side in irregular type (Fig. 5C). Contraction of cytoplasmic and nuclear contents formed compact and dense structure of apoptotic cells. Apoptotic cells showed alterations in mitochondria and cytoplasmic matrix (mx) as well (Fig. 5E–F). Necrotic changes such as plasma membrane disruption, lysed cytoplasmic matrix and nuclear debris were seen in Pm LPS-treated cells (Fig. 5B–F, asterisk).

LPS-treated macrophages showed many ultrastructural changes including nuclear condensation, chromatin margination and mitochondrial damage in comparison to control cells (Fig. 6A–D). Aggregates of LPS in bilayer vesicles were seen within a phagocytic vacuole in cytoplasm of the macrophages (Fig. 6B, arrow). Nuclear fragmentation, micronuclei formation, electron dense cytoplasmic inclusion and mitochondrial swelling were consistently observed in macrophages (Fig. 6B–C). Both types of chromatin margination, as described in lymphocytes, were observed in LPS-treated macrophages. Necrotic changes such as plasma membrane disruption, cell debris and nuclear

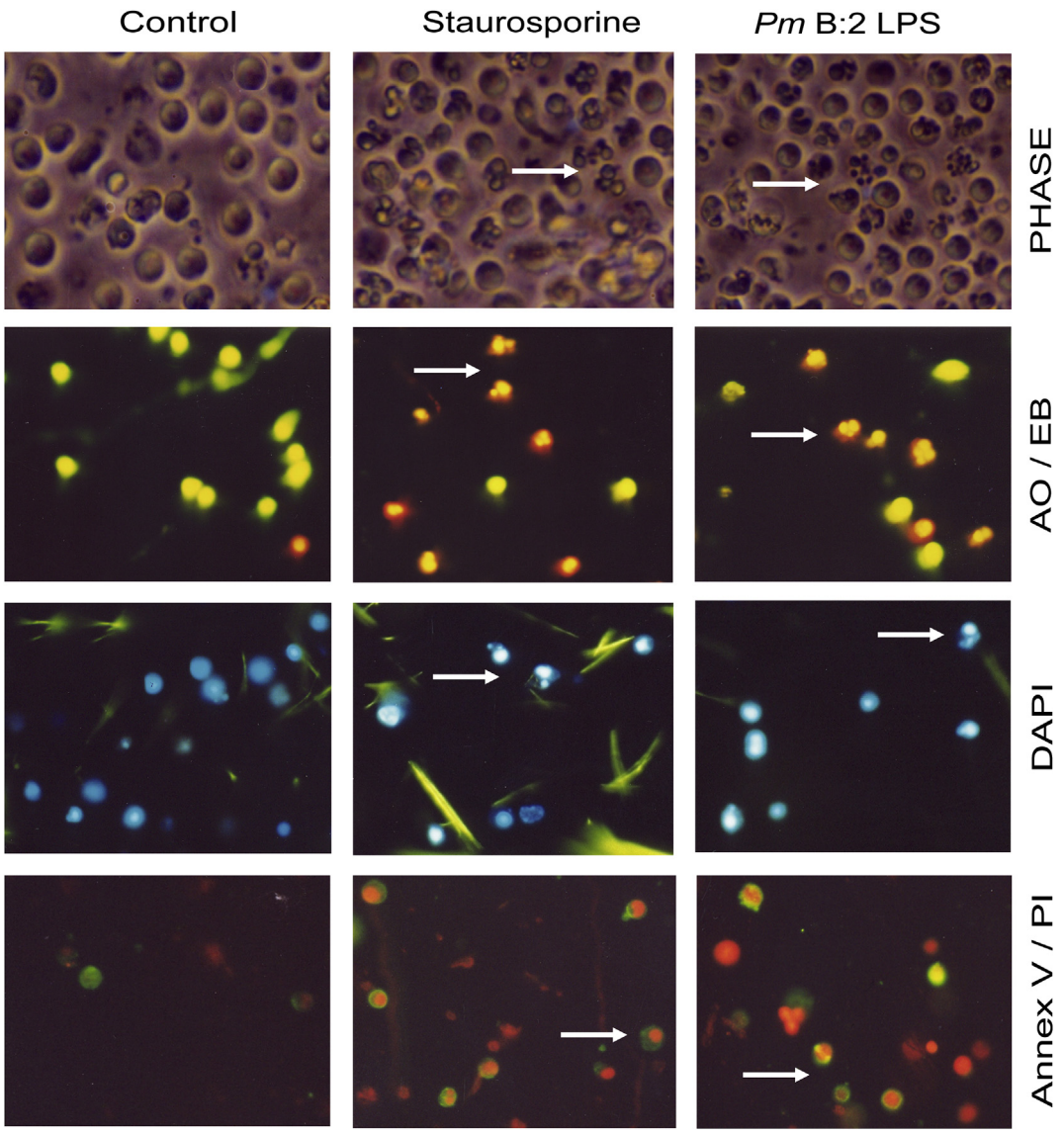


**Fig. 3.** Microscopic detection of cell death in bovine lymphocytes. Control or Pm B:2 LPS (10 μg/ml for 12 h time-point)-treated cells were analyzed for fragmented nuclear structure (arrow) using AO/EB, DAPI or Annexin V/PI staining (200×). Representative images were shown from three independent experiments.

fragmentation were seen in macrophages (Fig. 6B–C, asterisk). Apoptotic bodies containing condensed nuclear materials were connected by thin cell membrane (Fig. 6D).

LPS-treated neutrophils showed apoptotic changes like chromatin condensation and margination. Some neutrophils showed secondary necrotic changes such as fragmented nuclei with complete loss of cytoplasmic matrix (Fig. 6E). At places, apoptotic neutrophils contained membrane-bound vesicles containing nuclear elements and condensed nuclear fragments (Fig. 6F).

### 3.4. Caspases mediate Pm LPS induced-cell death in bovine leukocytes

Higher expression of pro-apoptotic gene (Bax) and increased caspase-3 activity were seen in Pm LPS-treated leukocytes (Supplemental Table 3). LPS-treated macrophages produced higher ($p < 0.05$) amount of nitric oxide (NO) when compared to control cells ($10.6 \pm 1.8$ *vs* $2.6 \pm 0.3$ μM). Pre-treatment with caspase-3 (Z-DEVD) inhibitor resulted in less number of apoptotic cells ($p < 0.05$) in LPS-treated lymphocytes (Fig. 7A), macrophages (Fig. 7B) and neutrophils (data not shown). Interestingly, pre-treatment with pan-caspase (Z-VAD) inhibitor showed more reduction in cell death (Fig. 7A–B). Also, pre-treatment with NO inhibitor (L-NAME) resulted in a 40% less apoptosis in macrophages (data not shown).

### 3.5. Mitochondrial dysfunction mediates Pm LPS-induced cell death in bovine leukocytes

Pm LPS-treated leukocytes had dissipated mitochondrial membrane potential (ΔΨm) when compared to untreated control cells (Supplemental Table 2). To test whether dissipated ΔΨm and mitochondrial permeability transition (MPT) initiate cell death, pre-treatment with CsA (MPT inhibitor) reduced ($p < 0.05$) apoptosis in LPS-treated lymphocytes (Fig. 8A), macrophages (Fig. 8B) and neutrophils (data not shown).

## 4. Discussion

The isolates of *P. multocida* cause septicemic and acute pulmonary infection characterized by necrotizing inflammation. Previous studies have reported that Pm LPS was a major virulence factor, as mutation in LPS structure prevented replication and survival of Pm in mammalian host [6–8]. Being an extracellular bacterium, exponential replication of

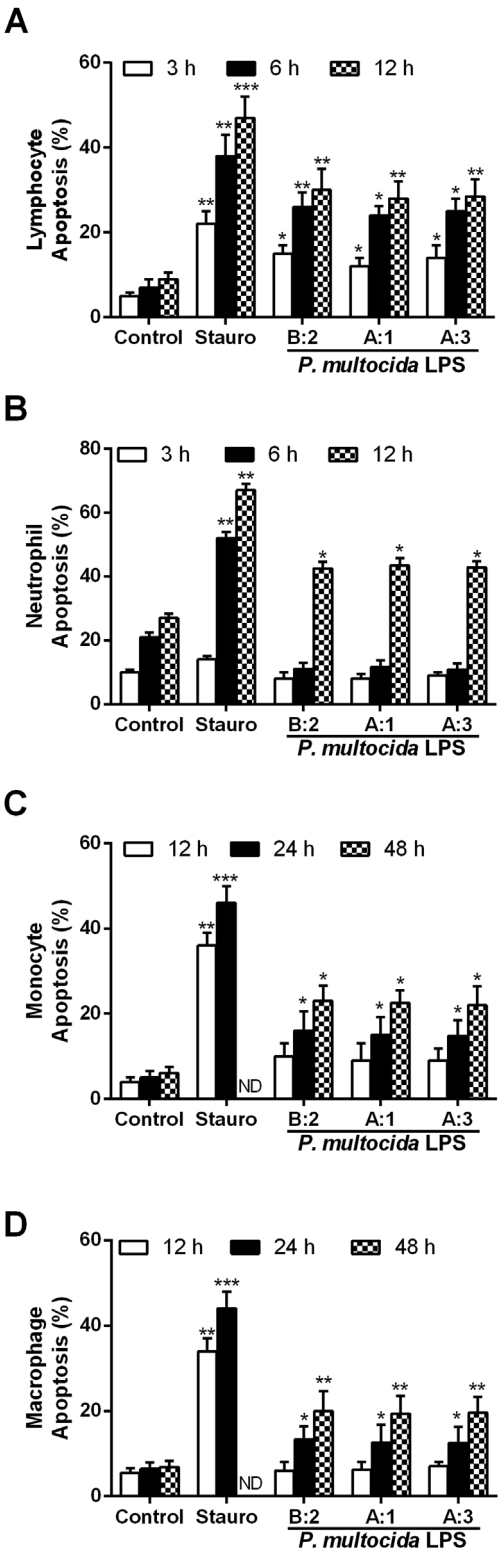


**Fig. 4.** Per cent apoptosis in (**A**) bovine lymphocytes, (**B**) neutrophils, (**C**) monocytes and (**D**) macrophages treated with Pm LPS (10 μg/ml) at different time-points. Data represent mean ± SD from three independent experiments (Student' *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001).

Pm in blood and tissue matrix could release a large amount of endotoxin (LPS), which can elicit protective immune response as to clear the infection. Alternatively, excessive activation of immune cells by LPS could aggravate the immunopathology of acute infections. However, it is unclear how the bovine leukocytes respond to Pm LPS. In this study, we investigated *in vitro* responses of bovine leukocyte to Pm LPS and observed many of the commonly known cellular responses of endotoxin such as cell proliferation and activation of inflammatory genes. Importantly, Pm LPS also induced cell death in leukocytes underlining a pathogenic role of endotoxin during acute pasteurellosis [4,6,25]. Thus, as in other Gram-negative bacterial infections, elevated levels Pm LPS could likely activate excessive inflammation, host cell death and tissue damage leading to acute death during pasteurellosis [5,16,20,23]. Further, the results of this study suggest that Pm LPS elicit a bi-phasic or bi-functional response in which LPS at certain threshold level can activate cell proliferation and cytokine-mediated inflammation, while at higher levels induce cell death and tissue pathology. Although, cellular activation by Pm LPS results in cytokine production and protective inflammatory changes, the induction of cell death could result in loss of immunocompetent cells favoring unfettered bacterial replication [4,19,20,23,35]. Importantly, LPS-induced immune cell death could also result in collateral tissue damage, aggravating sepsis-like response during acute phase of infection [4,36]. Thus, immuno-pathological changes of acute pasteurellosis could result as combined effects of bacterial products and host inflammatory factors [4].

Typical nuclear and cellular changes consistent with apoptosis and/or necrosis were seen in bovine leukocytes exposed to Pm LPS. These pathological processes suggest that Pm LPS is detrimental for host cells. Previous studies have reported that *M. haemolytica* LPS and LKT induced cell death in leukocytes and endothelial cells [19,22,25,28]. Results of this study also suggest that the kinetics of Pm LPS-induced cell death is different among subsets of leukocytes. Pm LPS caused a time-dependent and progressively increased apoptosis of lymphocytes, while a delay in apoptosis of neutrophils at early time-point. In contrast, Pm LPS caused differentiation and activation of monocytes and macrophages at early time-points, but induced apoptosis and necrosis at later time-points. This heterogeneity in cell death process among leukocyte subsets could be a difference in activation state of intracellular proteins and cytokines in cell-specific milieu. The observations of higher expression of TNF-α gene in PBMC and increased NO production in macrophages suggest an involvement of autocrine cytokine networks for cell death [26]. To support this, a reduction in apoptosis was observed in macrophages that were pre-treated with NO-inhibitor, confirming a role for NO in cell death [26,37,38]. Although monocytes and macrophages were relatively resistant to cell death at initial time-points, the accumulation of TNF and NO could subsequently trigger cell death at later time-points. As well, LPS could likely activated pro-survival signals in neutrophils, as seen by lowered spontaneous apoptosis at initial time-points. However, a sustained LPS stimulation had overcome the survival signals and activated death signals in neutrophils at later-time points, leading to apoptosis and/or secondary necrosis [39–42]. On the other hand, lymphocytes are prone to LPS-mediated cell death as early as 3 h and higher numbers of apoptotic and/or necrotic cells were observed at 12 h. This suggests that exposure to LPS, but lack of pro-survival factors at early time point, could rapidly trigger cell death in lymphocytes. Further, the metabolic profiles of cells could determine the extent of cell death. Unlike neutrophils and lymphocytes, the differentiated macrophages with versatile metabolic pathway exhibit lesser frequency of cell death, suggesting their resilience to stress. Accordingly, macrophages could adapt metabolically better to handle death signals and may not activate inflammasome-mediated pyroptosis [43].

Although leukocyte subsets exhibited a different kinetics of cell death, Pm LPS triggered mitochondrial dysfunction and caspases that mediated death in leukocytes. The levels of active caspase-3, typical ultrastructural changes of nuclei and the effect of caspase inhibitors on kinetics of cell death suggest a caspase-dependent apoptosis in Pm LPs-treated cells [25,29,37]. Curiously, pan-caspase inhibitors prevented more cell death than that of caspase-3 inhibitor, suggesting a role for caspase-1 and/or caspase-11, which mediate pyroptosis following exposure to LPS [30–32]. Pyroptosis is a form of cell death, similar to

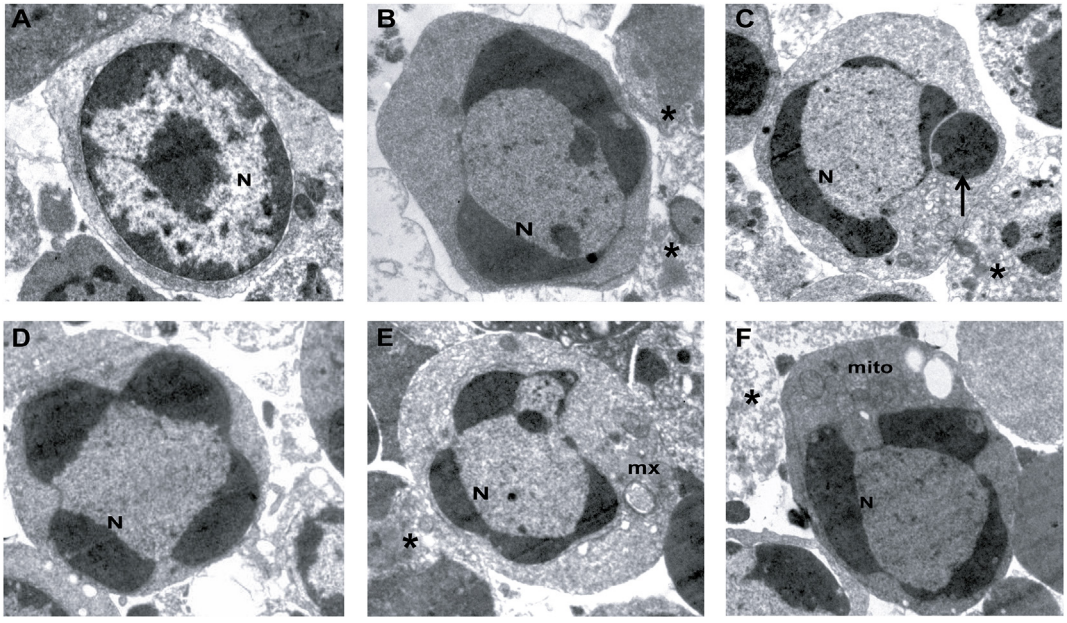


**Fig. 5.** Ultrastructural changes of bovine lymphocytes treated with Pm B:2 LPS for 12 h. (**A**) Control lymphocyte with normal cellular and nuclear (N) structures; (**B**) Apoptotic lymphocyte with condensed and crescent-shaped nucleus (N); (**C**) Apoptotic lymphocyte with condensed and horse shoe-shaped nucleus (N). Note round apoptotic micronuclei (arrow); (**D**) Apoptotic lymphocyte with marginated nuclear chromatin and fragmented nucleus (N); **(E)** Apoptotic lymphocyte with marginated nucleus (N) and disorganized cytoplasmic matrix (mx); (**F**) Apoptotic lymphocyte with fragmented nucleus (N) and swollen mitochondria (mito). Note necrotic changes such as plasma membrane disruption, lysed cytoplasmic matrix and nuclear debris (**B-F**, asterisk).

apoptosis, but activated by caspase-1 and 11, independent of caspase-3, 8 or 9 [44]. Activation of inflammasome facilitate processing of pro-caspase-1/11 to active caspase-1/11 that triggers pyroptosis, which shares morphological features of both apoptosis and necrosis [43]. The observation of ultrastructural changes consistent with necrotic changes in LPS-treated leukocytes suggests that Pm LPS could either directly activated necrosis or some of the apoptotic cells were undergone secondary necrosis [22,28]. Further, activation of inflammasome by intracellular LPS sensing results in pyroptosis, which could be seen as necrotic changes at ultrastructural level [32]. Alternatively, the localization of aggregates of LPS on internal organelles (such as mitochondria, Golgi body, nuclear membrane etc.) might disrupt cell structure and/or physiology leading to cell stress and cell death [24]. Consistently, aggregates of LPS in phagocytic vacuoles were seen in

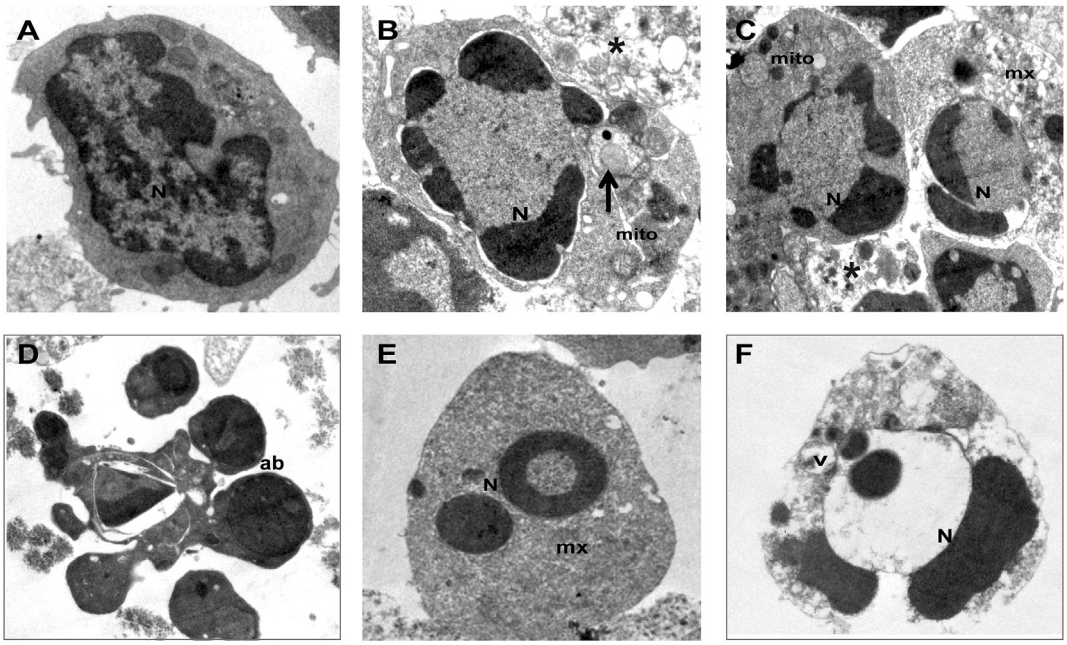


**Fig. 6.** Ultrastructural changes of bovine macrophages (48 h) and neutrophils (12 h) treated with Pm B:2 LPS. (**A**) Control macrophage showing normal cellular and nuclear (N) structures; (**B**) Apoptotic macrophage with marginated or fragmented nucleus (N) and swollen mitochondria (mito). Note aggregates of LPS in bilayer vesicles within a phagocytic vacuole in cytoplasm (arrow); (**C**) Apoptotic macrophages with condensed and crescent-shaped or fragmented nuclei (N), disorganized cytoplasmic matrix (mx) and swollen mitochondria (mito); Note necrotic changes like disrupted plasma membrane, fragmented nuclei and cell debris (**B-C**, asterisk); (**D**) Apoptotic macrophage showing apoptotic bodies (ab) connected by thin cell membrane; (**E**) Apoptotic neutrophil with fragmented nuclei (N) and completely disorganized cytoplasmic matrix (mx); (**F**) Apoptotic neutrophil with fragmented nuclei (N) and cytoplasmic vacuolation (v) and disorganization.

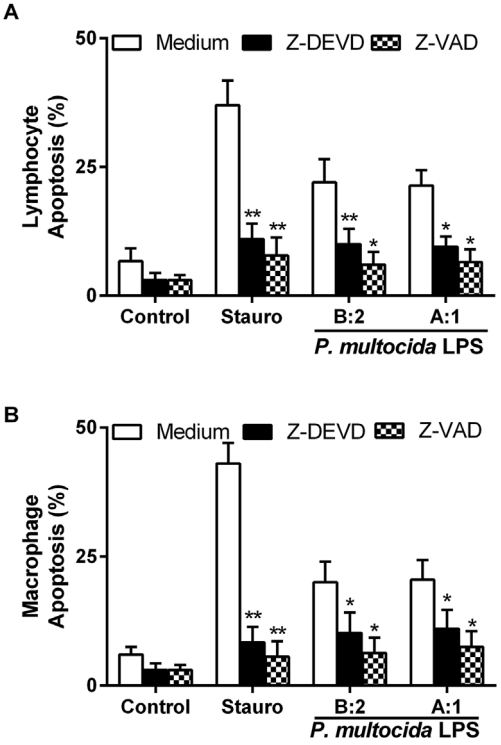


**Fig. 7.** Caspases mediate apoptosis in Pm LPS-treated bovine lymphocytes (**A**) and macrophages (**B**). Cells were untreated or treated with caspse-3 inhibitor (Z-DEVD) or pan-caspase inhibitor (Z-VAD) prior to LPS stimulation and per cent apoptosis was calculated at 6 h (lymphocytes and neutrophils) or 24 h (monocytes and macrophages). Data represent mean ± SD from three independent experiments (Student' *t*-test, *p < 0.05, **p < 0.01).

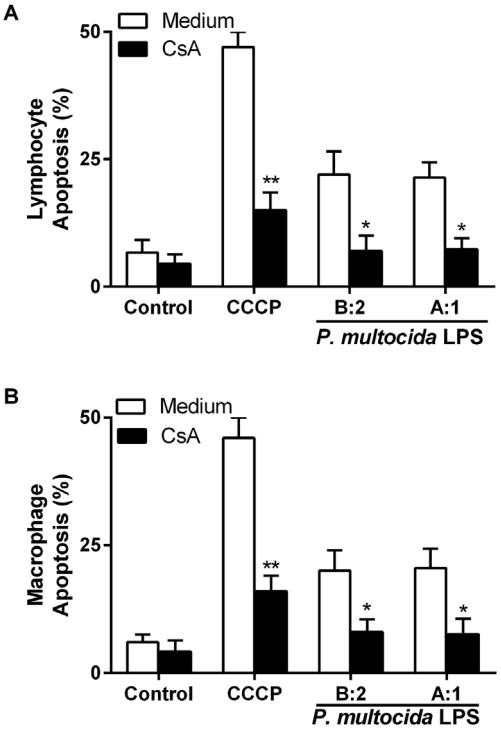


**Fig. 8.** Mitochondrial dysfunctions cause cell death in Pm LPS treated bovine lymphocytes (**A**) and macrophages (**B**). Cells were untreated or treated with MPT inhibitor (CsA) prior to LPS treatment and per cent apoptosis/necrosis was calculated at 6 h (lymphocytes) or 24 h (macrophages). Data represent mean ± SD from three independent experiments (Student' *t*-test, *p < 0.05).

association with and organelle damage at ultrastructural level in Pm LPS-treated leukocytes. Finally, LPS-treated leukocytes had a significant loss of mitochondrial membrane potential (ΔΨm) suggesting that endotoxin likely perturbed mitochondrial structure and function. Electron microscopic analysis confirmed the mitochondrial damage in these cells. In addition, level of ΔΨm dissipation corresponded to the frequency of apoptosis in these cells suggesting that release of mitochondrial enzymes (e.g., cytochrome-c) through MPT pores had activated the cell death process [23,27,45]. Supporting this notion, pre-treatment of cells with CsA (MPT inhibitor) inhibited cell death in LPS-treated leukocytes. Thus, mitochondrial dysfunction likely mediated cell death in LPS-treated leukocytes. Thus, Pm LPS induces cell death via more than two or three mechanisms. Future studies, using animal models of LPS administration, will explore the detailed mechanism of Pm LPS-mediated cell death and its contribution in immune-pathogenesis of acute pulmonary infections.

In conclusion, *in vitro* exposure of bovine leukocytes with Pm LPS induces cellular responses such as cell proliferation, cell activation and cell death.

## Conflicts of interest

Authors declares that they have no conflict of interest.

## Acknowledgements

This study was supported by the *All-India Network Project on Heamorrhagic Speticemia* (AINPHS02-07) from Indian Council of Agricultural Research. Authors thank Dr. A. A. Kumar, Principal Scientist, Division of Bacteriology for contributing bacterial isolates and technical suggestions. The authors thank the research technicians in the Divisions of Veterinary Pathology and Bacteriology for assisting in experiments.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2018.04.030.

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