Original article

Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-α, IL-1β, IL-12 and IFN-γ

Eun J. Sohn[a], Max J. Paape[b]*, Erin E. Connor[b], Douglas D. Bannerman[b], Raymond H. Fetterer[c], Robert R. Peters[a]

[a] Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, USA
[b] Bovine Functional Genomics Laboratory, USDA-ARS, Beltsville, MD 20705, USA
[c] Animal Parasitic Diseases Laboratory, USDA-ARS, Beltsville, MD 20705, USA

Abstract – After intramammary infection, polymorphonuclear neutrophil leukocytes (PMN) are the first cells recruited into the mammary gland. Rapid recruitment of and bacterial phagocytosis and killing by PMN are the most effective defenses against establishment of bacterial infection. In addition to their phagocytic and bactericidal properties, PMN may play a key supportive role through secretion of cytokines during the innate immune response. We sought to determine whether bovine PMN produce cytokines in response to stimulation by lipopolysaccharide (LPS). To investigate the effects of LPS on the expression of cytokines secreted by bovine PMN, we measured the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-12, and interferon (IFN)-γ by ELISA after stimulation with different concentrations of LPS, and secretion of IL-8 after co-stimulation with LPS and either TNF-α or IL-1β. Bovine PMN were shown to secrete TNF-α, IL-1β, IL-12, IL-8 and IFN-γ in response to LPS. Co-incubation of PMN with LPS and TNF-α increased secretion of IL-8 when compared to LPS alone. It was concluded that LPS stimulation up-regulates the secretion of cytokines by bovine PMN, and that co-incubation of LPS with TNF-α had an additive effect on the secretion of IL-8. These data show that bovine PMN, in addition to their phagocytic and bactericidal properties, may play a supportive role in the innate immune response to infection by Gram-negative bacteria through their ability to produce immuno-regulating cytokines.

lipopolysaccharide / bovine / neutrophils / cytokines / mastitis

1. INTRODUCTION

During intramammary infection polymorphonuclear neutrophil leukocytes (PMN) are recruited to the site of infection by chemotactic agents such as interleukin (IL)-8, IL-1β, tumor necrosis factor (TNF)-α and leukotriene-B4 released from activated endothelium and macrophages [9, 32]. In mammary quarters free from bacterial infection, macrophages are the predominant cell type (35–79%), followed by PMN (3–26%), lymphocytes (10–24%) and epithelial cells (2–15%) [27, 30]. Following intramammary infection or challenge with lipopolysaccharide (LPS), the percentage of PMN in milk can approach 100% and absolute number of PMN can reach 1×10^8/mL of milk [5,31].

* Corresponding author:
  mpaape@anri.barc.usda.gov

This enormous migration of PMN into tissue provides the first line of immune defense against bacterial infection.

PMN and mononuclear cells play important roles in the host defense against Gram-negative bacterial infection. Of all the clinical cases of bovine mastitis that occur annually, 40% are caused by Gram-negative bacteria [16, 46]. A key component of the host immune response to Gram-negative infections is the upregulation of cytokine production [5, 39, 40]. Most studies have focused on the immunomodulation changes affected by bovine mononuclear cells, and only a few have been conducted on immunomodulation changes affected by bovine PMN. This is partially attributed to the view that although PMN play a vital role in the inflammatory response through their phagocytic and bactericidal killing mechanisms, they are short-lived white blood cells that spontaneously undergo apoptosis [32].

Several cytokines have been proposed to be involved in mastitis, including IL-1β, IL-8, IL-12, interferon (IFN)-γ and TNF-α [37, 39], and are responsible for modulating the life span and physiological functions of PMN [19, 41]. IL-1β, IL-8, and IFN-γ, reportedly prolong human PMN survival [32]. TNF-α induces apoptosis in both human and bovine-derived PMN [32].

IL-1β and TNF-α contribute to the establishment of inflammation by altering vascular permeability, and promoting PMN recruitment to the site of infection by inducing vascular endothelial adhesion molecule expression. These two cytokines also increase hepatic synthesis of acute phase proteins that facilitate complement activation and host detection of bacterial cell wall products [15, 18]. PMN recruitment to the site of infection is further mediated by the upregulation of the chemoattractant IL-8 [40]. The concentration of IL-8 increases rapidly after experimental challenge with either LPS or *Escherichia coli*, and precedes the increase in milk somatic cells [5, 7]. IL-12, which is produced by macrophages and B-lymphocytes in response to LPS, plays a key role in the initiation of both innate and antibody-specific pro-inflammatory immunity [44]. IL-12 also enhances the cytotoxic activity of natural killer (NK) cells [2, 34]. Further, IL-12 contributes to the innate immune response by stimulating the production of IFN-γ, an activator of PMN and macrophages, and plays a central role in controlling the host's response to bacterial and viral infection [14]. IFN-γ can either enhance or suppress chemokine secretion in response to pro-inflammatory cytokines such as IL-1β and TNF-α. IFN-γ can also modulate chemokine receptor expression on endothelial cells and affect diapedesis of PMN into tissue [42].

The purpose of this study was to determine if bovine PMN produce IL-8, IL-1β, IL-12, TNF-α and IFN-γ in response to LPS.

2. MATERIALS AND METHODS

2.1. Cows

Ten clinically normal mid- to late-lactating Holstein cows were selected from the USDA Beltsville Agricultural Research Center (BARC) dairy herd. The use and care of all animals were approved by the Beltsville Agricultural Research Center and the University of Maryland Animal Care and Use Committees.

2.2. Blood sampling and PMN isolation

Blood was collected from the tail vein by venipuncture in heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). PMN were isolated using the procedure of Hahn and Tolle [21]. Blood was centrifuged at $500 \times g$, for 5 min, at 4 °C. Plasma, buffy coat and 1/3 of

the red blood cells (RBC) were removed. The remaining RBC and white blood cells (WBC) (10 mL) were suspended dropwise into a double volume of cold 0.2% NaCl solution and gently mixed for about 1 min for lysis of RBC. Immediately, half the original volume of cold 3.7% NaCl solution (5 mL) was added to restore isotonicity. The suspension was centrifuged at $200 \times g$, for 1 min, at 4 °C. The WBC pellet was washed twice with 20 mL of 0.0132 M, pH 7.4, 0.85% NaCl solution (phosphate-buffered saline solution; PBS) (BioWhittaker, Walkersville, MD, USA). After enumeration by an electronic cell counter (Beckman Coulter Multisizer II, Miami, FL, USA), PMN were adjusted to 5×10^6/mL in RPMI medium 1640 with L-glutamine and without phenol red (Invitrogen Corp., Grand Island, NY, USA). Smears were prepared from the pellet on glass microscope slides and stained with Hemacolor (Merck, D-64293 Darmstadt 1, Germany) in a automatic slide stainer (Hema-Tex 2000, Bayer, Corp. Elkhart, IN, USA). Differential microscopic counts were determined by counting 100 cells. The cell preparation consisted of 93% PMN, 4% eosinophils, 2% monocytes and 1% lymphocytes. Viability of PMN, as determined by exclusion of trypan blue, was 98%.

2.3. PMN culture and activation

PMN were added to 96-well tissue culture plates (100 μL/well), and incubated at 37 °C, in a 5% CO_2 incubator for 18 h. To study the release of IL-8, PMN ($n = 5$ cows) were cultured with either media alone, human TNF-α (10 ng/mL) (Endogen, Inc., Woburn, MA, USA), bovine IL-1β (10 ng/mL) (Endogen), LPS (0.1 μg/mL, derived from *E. coli* 0111:B4, Sigma Chemical Co., St. Louis, MO, USA), or the combination of LPS (0.1 μg/mL) and either human TNF-α

(10 ng/mL) or bovine IL-1β (10 ng/mL). To study the release of IFN-γ, IL-1β, IL-12, and TNF-α, PMN ($n = 10$ cows) were cultured at 37 °C in a 5% CO_2 incubator for 18 h with 0, 1, 10 or 100 μg/mL of LPS. All supernatants were collected after centrifugation (9 300 × g, 5 min, 4 °C) and stored at 20 °C for further processing.

2.4. Quantification of IL-8

IL-8 was determined in undiluted PMN supernatants using a commercially available human IL-8 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). The antibody pairs used in this kit have been previously shown to cross-react with bovine IL-8 [39, 40]. The optical density at 450 nm and a correction wavelength of 550 nm was determined on a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Values, expressed in pg/mL, were interpolated from a standard curve of known amounts of human IL-8 using linear regression analysis.

2.5. Quantification of IFN-γ

IFN-γ was determined in undiluted supernatants obtained from cultures of 0.5×10^6 PMN with a commercially available bovine IFN-γ ELISA kit (Biosource/Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Recombinant bovine IFN-γ (Serotec, Inc., Raleigh, NC, USA) was used to generate a standard curve for the ELISA. OD at 450 nm and a correction wavelength 550 nm were measured on a microplate reader (Bio-Tek Instruments).

2.6. Quantification of IL-12

Flat-bottom 96-well plates were coated overnight at 4 °C with 4 μg/mL of mouse

anti-bovine IL-12 (Serotec) antibody diluted in 0.05 M sodium carbonate, pH 9.6. The plates were washed three times with 0.05% Tween-20 diluted in 50 mM Tris buffer saline (TBS, Sigma Chemical Co.), pH 8.0, and blocked with 2% fish skin gelatin (Sigma Chemical Co.) for 1 h at room temperature. The plates were washed and 100 μL of sample was added to the anti-IL-12-coated plates. Following a 2-h incubation at room temperature, the plates were washed, and 100 μL of biotin-conjugated mouse anti-bovine IL-12 antibody (Serotec) diluted to 1 μg/mL was added to each well. The plates were incubated for 1 h at room temperature and washed with TBS. HRP-conjugated strepavidin (Sigma Chemical Co.) was diluted 1:500 in TBS wash buffer containing 0.2% gelatin, and 100 μL of this solution was added to each well. The plates were incubated for 1 h at room temperature and washed. Trimethylbenzidine (TMB) substrate solution (100 μL, Sigma Chemical Co.) was added to each well. The reaction was stopped by the addition of 100 μL of 2M H_2SO_4 and the absorbance was read at 450 nm on a microplate reader (Bio-Tek Instruments). A standard curve of known amounts of recombinant human IL-12 (Serotec) were assayed in parallel.

2.7. Quantification of IL-1β and TNF-α

Flat bottom 96-well plates were coated overnight at 4 °C with 5 μg/mL of mouse anti-bovine IL-1β antibody (Serotec) or with 100 μL of anti-mouse TNF-α mAb [31] diluted 1:2000 in 0.05 M sodium carbonate, pH 9.6. The plates were washed three times with 0.05% Tween 20 diluted in 50 mM TBS, pH 8.0, and blocked with 2% fish skin gelatin (Sigma Chemical Co.) for 1 h at room temperature. The plates were washed, and 100 μL of sample was added to the anti-IL-1β or anti-TNF-α-coated plates. Following a 2-h

incubation at room temperature, the plates were washed, and either 100 μL of rabbit polyclonal anti-bovine IL-1β antibody (Serotec) diluted to 1:500, or 100 μL rabbit polyclonal anti-bovine TNF-α antibody [31] diluted 1:20 000 was added. The plates were incubated for 1 h at room temperature and washed. HRP-conjugated anti-rabbit IgG (Sigma Chemical Co.) was diluted 1:5000 in TBS wash buffer containing 0.2% gelatin, and 100 μL of this solution was added to each well. The plates were incubated for 30 min at room temperature and washed. TMB substrate solution (Sigma Chemical Co.) was added to each well. The reaction was stopped by the addition of 100 μL of 2 M H_2SO_4 and the absorbance was read at 450 nm on a microplate reader. Values were interpolated from standard curves of known amounts of recombinant bovine IL-1β (Pierce) and recombinant bovine TNF-α (Genetech Corp., South San Francisco, CA, USA).

2.8. Statistical methods

Data were analyzed by one way analysis of variance (ANOVA) (Prism version 4.0 for Windows; Graph Pad Software Inc., San Diego, CA, USA). The Tukey post-hoc comparison test was used to assess differences between individual treatment groups and the respective media control. A P-value of < 0.05 was considered significant.

3. RESULTS

3.1. Secretion of IL-8 by PMN

Neither TNF-α nor IL-1β elicited ($P > 0.05$) release of IL-8 from PMN when compared to media alone, although IL-8 levels were slightly elevated in the supernatants of PMN stimulated with TNF-α or IL-1β (5.68 ± 2.5, 14.3 ± 7.4 pg/mL)
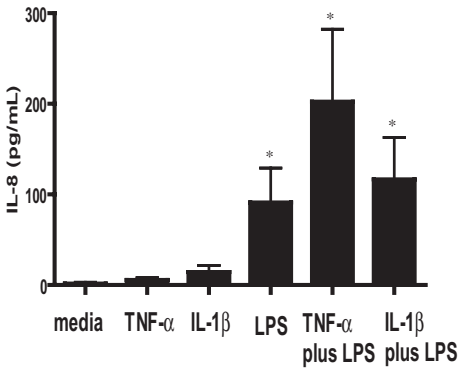
Figure 1. The effect of different stimuli on IL-8 release from bovine PMN. PMN (5×10^5 cells) were incubated with either RPMI, TNF-α (10 ng/mL), IL-1β (10 ng/mL), LPS (0.1 μg/mL), TNF-α (10 ng/mL) + LPS (0.1 μg/mL) or IL-1β (10 ng/mL) + LPS (0.1 μg/mL). Results are expressed as means with S.E.M. (n = 5 cows). * Significantly different ($P < 0.05$) from media control.

Figure 2. Concentration of IFN-γ in supernatants after stimulation of PMN with LPS. PMN were plated at 5×10^5 cells/well in 96 well plates and were stimulated with different concentrations of LPS (n = 10 cows). * Significantly different ($P < 0.05$) from media control. Results are expressed as means with S.E.M.

(Fig. 1). In the presence of 0.1 μg/mL LPS, PMN exhibited enhanced ($P < 0.05$) IL-8 release, averaging 91.4±38 pg/mL. Adding LPS together with TNF-α further increased ($P < 0.05$) IL-8 release (202 ± 80 pg/mL). The increase in IL-8 observed with LPS plus IL-1β (116.7 ± 46 pg/mL) was not different from that observed with LPS alone ($P > 0.05$).

3.2. Secretion of IFN-γ by PMN

The concentration of IFN-γ (71 ± 46 pg/mL) secreted by unstimulated control PMN was similar to that observed for PMN incubated with either 1 or 10 μg/mL of LPS (Fig. 2). Secretion of IFN-γ increased ($P < 0.05$) (162 ± 48 pg/mL) in the presence of 100 μg/mL of LPS.

3.3. Secretion of IL-1β by PMN

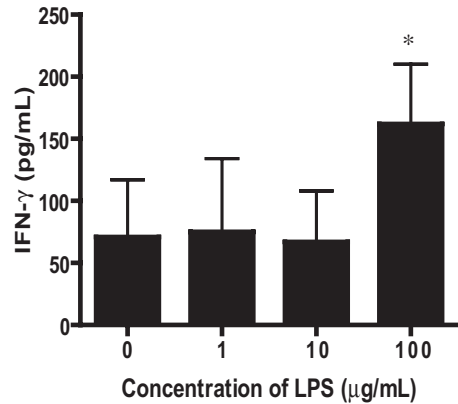IL-1β increased ($P < 0.01$) in supernatants derived from PMN exposed

to different concentrations (1, 10 and 100 μg/mL) of LPS when compared to media control (Fig. 3). Similar increases were observed for all three concentrations of LPS.

3.4. Secretion of IL-12 by PMN

PMN secretion of IL-12 increased ($P < 0.05$) after incubation with either 1 or 10 μg/mL of LPS when compared to media control (Fig. 4). The increase observed with 100 μg/mL of LPS was more variable when compared to the other concentrations of LPS, and was not different ($P > 0.05$) from media control.

3.5. Secretion of TNF-α by PMN

Secretion of TNF-α was elevated ($P < 0.05$) in PMN culture media containing 1, 10 and 100 μg/mL of LPS when compared to unstimulated PMN (0.75 ± 0.05 ng/mL) (Fig. 5).
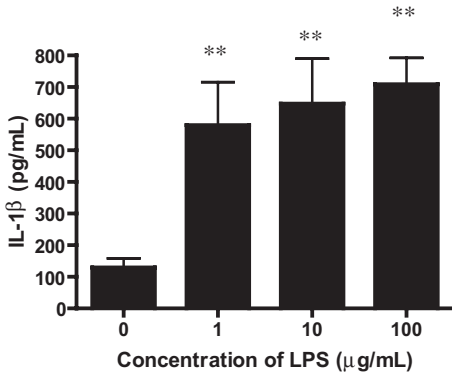
Figure 3. Concentration of IL-1β in supernatants after stimulation of PMN with LPS. PMN were plated at 5×10^5 cells/well in 96 well plates and were stimulated with different concentrations of LPS ($n = 10$ cows). ** Significantly different ($P < 0.01$) from media control. Results are expressed as means with S.E.M.

Figure 5. Concentration of TNF-α in supernatants after stimulation of PMN with LPS. PMN were plated at 5×10^5 cells/well in 96 well plates and were stimulated with different concentrations of LPS ($n = 10$ cows). * Significantly different ($P < 0.05$) from media control. Results are expressed as means with S.E.M.
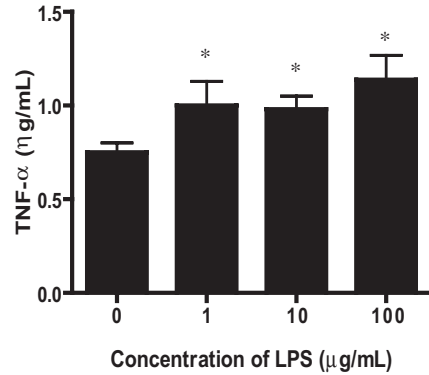

Figure 4. Concentration of IL-12 in supernatants after stimulation of PMN with LPS. PMN were plated at 5×10^5 cells/well in 96 well plates and were stimulated with different concentrations of LPS ($n = 10$ cows). * Significantly different ($P < 0.05$) from media control. Results are expressed as means with S.E.M.
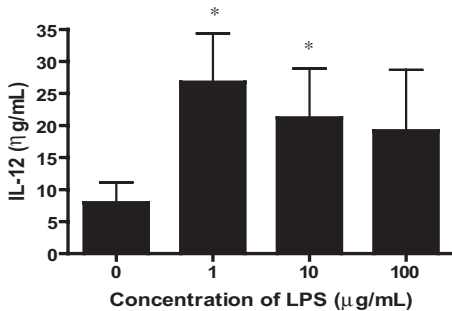
4. DISCUSSION

The results of this study demonstrate that circulating bovine PMN respond to LPS stimulation by secreting TNF-α, IL-1β, IFN-γ, IL-8 and IL-12, and contribute to the innate immune response in dairy cows. In the present study, PMN were cultured in RPMI medium without fetal bovine serum (FBS) for 18 h. Microscopic observations of the plates indicated that the PMN were adherent to the bottom of the wells and flattened, which could have a stimulatory effect on PMN [28]. This activation could account for the presence of IFN-γ, IL-1β, IL-12 and TNF-α in the media control wells. To evaluate the direct effects of LPS on the PMN, PMN were stimulated in the absence of endogenous mediators such as CD14 and LBP which are present in FBS. This, however, necessitates the use of higher concentrations of LPS to activate these cells. Because 2% of the isolated cells were monocytes (10 000/well), it is conceivable that the contaminating monocytes may have contributed, in part, to the cytokine responses observed in the present study. In one study, 80 000 monocytes/well stimulated with concanavalin A for 48 h, produced a TNF-α response similar to what we observed after stimulation of 500 000PMN/well with LPS for 18 h [8]. Thus, approximately 10%, or 0.1 ηg of TNF-α could have been secreted by the contaminated monocytes in our PMN

preparation. On a per cell basis the monocytes in the previous study [8] produced far more molecules of cytokines than the PMN in our study. However, during mastitis PMN far outnumber macrophages by several orders of magnitude, and can therefore be major sources of cytokines like TNF-α. It was recently demonstrated by real-time PCR that mRNA expression of TNF-α, IL-6, IFN-γ, IL-8 and IL-12 was up-regulated in milk PMN following experimental intramammary injection of either *E. coli, Staphylococcus aureus* or *S. aureus* α-toxin [3, 24, 37, 38]. Combined with the results from the present study it would appear that increased mRNA expression in milk PMN correlates with cytokine secretion.

All of the cytokines studied in this study have been reported to increase in milk after experimental intramammary infection by a variety of mastitis pathogens [6, 7, 34]. Further, these cytokines play key regulatory roles in the inflammatory response to bacterial pathogens. TNF-α has been reported to stimulate IL-8 release by human PMN [43]. In the present study TNF-α alone had no effect on IL-8 secretion by bovine PMN (Fig. 1). However, in combination with LPS, TNF-α produced an additive effect on IL-8 release by PMN when compared with only LPS. Thus, it appears that these two inflammatory agents cooperate to stimulate release of IL-8, and may contribute to defense of the mammary gland by promoting more rapid recruitment of PMN. Another example of cooperation between inflammatory mediators was reported by Rainard et al. [35], who showed that priming of bovine PMN with the chemoattractant C5a^{desArg} and TNF-α led to more efficient phagocytosis and killing of *S. aureus,* when compared with either C5a^{desArg} or TNF-α. In addition to its ability to enhance PMN secretion of IL-8, TNF-α was also reported to increase phagocytosis, degranulation and oxidative burst activity of bovine PMN, as well as enhanced migration through endothelium due to up-regulation of endothelial adhesion molecules [32]. It was also reported that bovine peripheral PMN possessed receptors for TNF-α, and expression of TNF-α receptors was regulated by other cytokines such as IFN-γ [29].

The results of the present study also show that in addition to LPS induced secretion of IL-8 by bovine PMN, secretion of TNF-α, IL-1β, IFN-γ and IL-12 by bovine PMN was sensitive to induction by LPS. Of the cytokines that were increased, IFN-γ and IL-1β were up-regulated to the greatest extent in response to LPS. However, unlike responses observed for TNF-α, IL-1β and IL-12, there was no IFN-γ response after stimulation with 1 and 10 μg of LPS. IFN-γ is produced by phagocytic cells such as NK cells, that play a major role in killing pathogen-infected cells and tumors [14]. However, overproduction of IFN-γ can produce dangerous side effects like autoimmune diseases [45]. How the secretion of IFN-γ is controlled is not known. Our results suggest that bovine PMN may control release of IFN-γ by requiring a higher threshold of activation by LPS when compared to other cytokines. The ability of PMN to produce IFN-γ points to a new role for bovine PMN during the innate immune response. Human PMN contain a small store of IFN-γ and this store is rapidly secreted upon stimulation by degranulating agents such as formyl peptides. After stimulation with LPS, human PMN synthesize IFN-γ [18]. This is consistent with our findings that bovine PMN also secrete IFN-γ after LPS stimulation. IL-1β can stimulate T-helper cells and B-cells to synthesize immunoglobulin [36]. It also promotes the adhesion of PMN, monocytes, T-cells, and B-cells to the endothelium by enhancing expression of adhesion molecules like intracellular adhesion molecule and endothelial leukocyte adhesion molecule, as well as proliferation and activation of NK cells [24].

IL-12 is chemotactic for human PMN, and also activates IFN-γ, IL-8 and TNF-α synthesis [1, 17]. The biological activities of IL-12 include enhancement of cytotoxic T cell and lymphokine-activated killer cell generation and activation, increased natural killer cell cytotoxicity, induction of activated T-cell and NK-cell proliferation, induction of IFN-γ production by NK cells and T cells, and inhibition of IgE synthesis by IL-4-stimulated lymphocytes via IFN-γ-dependent and -independent mechanisms [20, 22, 23, 45].

Elevated levels of IL-12 were detected in milk following experimental intramammary infection with either *Serratia marcescens, Streptococcus uberis*, *Staphylococcus* or *E. coli* [6, 7]. In those studies, the concentration of IL-12 in milk closely followed the milk somatic cell count, of which > 95% are neutrophils [27, 30, 32]. Based on the results of the present study that PMN are capable of producing IL-12, it may be hypothesized that the major source IL-12 in milk following experimental intramammary challenge are milk PMN.

Several studies showed that human PMN produced TNF-α, IL-12 and IL-8 after stimulation with microbial products such as bacterial LPS, *Toxoplasma gondii* and *Candida albicans* [10–12]. While human PMN normally undergo rapid apoptosis during culture, several inflammatory cytokines (e.g., IL-1β, TNF-α, IL-6, G-CSF, GM-CSF) prolong their life span [4, 13]. In vivo studies have demonstrated that the life span of bovine PMN from mammary quarters infected with *E. coli* is prolonged when compared to noninfected quarters [25]. Further, higher pre-infection viability of mammary PMN correlated with increased chemiluminescence activity, a more rapid bacterial clearance and recovery of infected quarters [25, 26].

Previous studies have demonstrated that mammary epithelial cells and mononuclear WBC, upon interaction with micro-bial pathogens, were able to generate a wide variety of cytokines [33]. To date, no one has demonstrated LPS induced cytokine secretion from bovine PMN. That bovine PMN are capable of producing several major inflammatory cytokines in response to LPS suggests that PMN may be important in directing early cell trafficking and cytokine-producing activities during infection by microbial pathogens. In conclusion, the results from the present study show that bovine PMN, in addition to their phagocytic and bactericidal properties, may play a supportive role in the innate immune response to infection by Gram-negative bacteria, through their ability to produce immuno-regulating cytokines.

REFERENCES

[1] Al-Mohanna F., Saleh S., Parhar R.S., IL-12 dependent nuclear factor-kappa B activation leads to de novo synthesis and release of IL-8 and TNF-α in human neutrophils, J. Leukoc. Biol. (2002) 72:995–1002.

[2] Allavena P., Paganin C., Zhou D., Sozzani S., Mantovani A., Interleukin-12 is chemotactic for NK cells and stimulates their interaction with vascular endothelium, Blood (1994) 84:2261–2268.

[3] Alluwaimi A.M., Leutenegger C.M., Farver T.B., Rossitto P.V., Smith W.L., Cullor J.S., The cytokine markers in S*taphylococcus aureus* mastitis of bovine mammary gland, J. Vet. Med. B Infect. Dis. Vet. Public Health (2003) 50:105–111.

[4] Bannerman D.D., Tupper J.C., Kelly J.D., Winn R.K., Harlan J.M., The Fas-associated death domain protein suppresses activation of NF-kappa B by LPS and IL-1 beta, J. Clin. Invest. (2002) 1093:419–425.

[5] Bannerman D.D., Paape M.J., Hare E.R., Sohn E.J., Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge, J. Dairy Sci. (2003) 86:3128–3137.

[6] Bannerman D.D., Paape M.J., Goff J.P., Kimura K., Lippolis J.D., Hope J.C., Innate immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*, Vet. Res. (2004) 35:681–700.

[7] Bannerman D.D., Paape M.J., Lee J.W., Zhao X., Hope J.C., Rainard P., *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection, Clin. Diagn. Lab. Immunol. (2004) 11:463–472.

[8] Burton J.L., Nonnecke B.J., Dubeski P.L., Elsasser T.H., Mallard B.A., Effects of supplemental chromium on production of cytokines by mitogen-stimulated bovine peripheral blood mononuclear cells, J. Dairy Sci. (1996) 79:2237–2246.

[9] Butcher C., Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity, Cell (1991) 67:1033–1036.

[10] Cacalano G., Lee J., Kikly K., Ryan A.M., Pitts-Meek S., Hultgren B., Wood W.I., Moore M.W., Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog, Science (1994) 29:590–591.

[11] Cassatella M.A., The production of cytokines by polymorphonuclear neutrophils, Immunol. Today (1995) 16:21–26.

[12] Cassatella M.A., Meda L., Gasperini S., D'Andrea A., Ma X., Trinchieri G., Interleukin-12 production by human polymorphonuclear leukocytes, Eur. J. Immunol. (1995) 25:1–5.

[13] Colotta F., Re F., Polentarutti N., Sozzani S., Mantovani A., Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products, Blood (1992) 80:2012–2020.

[14] Decker T., Stockinger S., Karaghiosoff M., Müller M., Kovarik P., IFNs and STATs in innate immunity to microorganisms, J. Clin. Invest. (2002) 109:1271–1277.

[15] Dinarello C.A., Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock, Chest (1997) 112:321–329.

[16] Erskine R.J., Tyler J.W., Riddell M.J., Wilson R.C., Theory, use, and realities of efficacy and food safety of antimicrobial treatment of acute coliform mastitis, J. Am. Vet. Med. Assoc. (1991) 198:980–984.

[17] Ethuin F., Gerard B., Benna J.E., Boutten A., Gougereot-Pocidalo M.A., Jacob L., Chollet-Martin S., Human neutrophils produce interferon gamma upon stimulation by interleukin-12, Lab. Invest. (2004) 84:1363–1371.

[18] Feghali C.A., Wright T.M., Cytokines in acute and chronic inflammation, Front. Biosci. (1997) 2:12–26.

[19] Foxman E.F., Campbell J.J., Butcher E.C., Multistep navigation and the combinational control of leukocyte chemotaxis, J. Cell Biol. (1997) 139:1349–1360.

[20] Gately M.K., Wolitzky A.G., Quinn P.M., Chizzonite R., Regulation of human cytolytic lymphocyte responses by interleukin-12, Cell. Immunol. (1992) 143:127–142.

[21] Hahn G., Tolle A., Comparative studies to characterize human and bovine group B-Streptococci (*Str. agalactiae*) by means of a bactericidal assay, Zentralbl. Bakteriol. A (1981) 249:15–23 (in German).

[22] Kiniwa M., Gately M., Gubler U., Chizzonite R., Fargeas C., Delespesse G., Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes, J. Clin. Invest. (1992) 90:262–266.

[23] Lee A., Whyte M.K., Haslett C., Inhibition of apoptosis and prolongation of neutrophil function longevity by inflammatory mediators, J. Leukoc. Biol. (1993) 54:283–288.

[24] Lee J.W., Bannerman D.D., Paape M.J., Huang M.-K., Zhao X., Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR, Vet. Res. (2006) 37:219–229.

[25] Mehrzad J., Duchateau L., Burvenich C., Viability of milk neutrophils and severity of bovine coliform mastitis, J. Dairy Sci. (2004) 87:4150–4162.

[26] Mehrzad J., Duchateau L., Burvenich C., High milk neutrophil chemiluminescence limits the severity of bovine coliform mastitis, Vet. Res. (2005) 36:101–116.

[27] Miller R.H., Paape M.J., Fulton L.A., The relationship of milk somatic cell count to milk yield for Holstein heifers after first calving, J. Dairy Sci. (1993) 6:728–733.

[28] Nathan C., Neutrophils and immunity: challenges and opportunities, Nat. Rev. Immunol. (2006) 6:173–182.

[29] Ohmann H.B., Campos M., McDougall L., Lawman M.J.P., Babiuk L.A., Expression of tumor necrosis factor-α receptors on bovine macrophages, lymphocytes and polymorphonuclear leukocytes, internalization of receptor-bound ligand, and some functional effects, Lymphokine Res. (1990) 9:43–58.

[30] Östensson K., Hageltorn M., Aström G., Differential cell counting in fractions collected milk from dairy cows, Acta Vet. Scand. (1998) 29:493–500.

[31] Paape M.J., Rautiainen P.M., Lilius E.M., Malstrom C.E., Elsasser T.H., Development of anti-bovine TNF-alpha mAb and ELISA for quantitating TNF-α in milk after intra-mammary injection of endotoxin, J. Dairy Sci. (2002) 85:765–773.

[32] Paape M.J., Bannerman D.D., Zhao X., Lee J.W., The bovine neutrophil: Structure and function in blood and milk, Vet. Res. (2003) 34:597–627.

[33] Rabinowich H., Herbeman R.B., Whiteside T.L., Differential effects of IL-12 and IL-2 on expression and function of cellular adhesion molecules on purified human NK cells, Cell. Immunol. (1993) 152:481–498.

[34] Rainard P., Riollet C., Innate immunity of the bovine mammary gland, Vet. Res. (2006) 37:369–400.

[35] Rainard P., Riollet C., Poutrel B., Paape M.J., Phagocytosis and killing of *Staphylococcus aureus* by bovine neutrophils after priming by tumor necrosis factor-α and the des-arginine derivative of C5a, Am. J. Vet. Res. (2000) 61:951–959.

[36] Reddy P.J., Biecha F., Minocha H.C., Anderson G.A., Morrill J.L., Fedorka-Cray P.J., Baker P.E., Bovine recombinant interleukin-2 augments immunity and resistance to bovine herpesvirus infection, Vet. Immunol. Immunopathol. (1989) 30:61–74.

[37] Riollet C., Rainard P., Poutrel B., Cells and cytokines in inflammatory secretion of bovine mammary gland, Adv. Exp. Med. Biol. (2000) 480:247–258.

[38] Riollet C., Painard P., Poutrel B., Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with *Staphylococcus aureus* alpha-toxin, Inflamm. Res. (2000) 49:486–496.

[39] Shuster D.E., Kehrli M.E., Stevens M.G., 1993. Cytokine production during endotoxin – induced mastitis in lactating dairy cows, Am. J. Vet. Res. (1993) 54:80–85.

[40] Shuster D.E., Kehrli M.E., Rainard P., Paape M.J., Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infections with *Escherichia coli*, Infect. Immun. (1997) 65:3286–3292.

[41] Springer T.A., Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm, Cell (1994) 76:301–314.

[42] Struyf S., Van Collie E., Paemen L., Put W., Lenaerts J.P., Proost P., Opdenakker G., Van Damme J., Synergistic induction of MCP-1 and-2 by IL-1β and interferon's in fibroblasts and epithelial cells, J. Leukoc. Biol. (1998) 63:364–372.

[43] Trine L., Bjarne O., The effect of TNF-α, PMA, and LPS on plasma and cell- associated IL-8 in human leukocytes, Thromb. Res. (2004) 113:75–83.

[44] Trincheri G., Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity, Annu. Rev. Immunol. (1995) 13:251–276.

[45] Yu J., Becknell B., Trotta R., Liu S., Boyd Z., Jaung M.S., Blaser B.W., Sun J., Benson D.M., Mao H., Yokohama A., Bhatt D., Shen L., Davuluri R., Weinstein M., Marcucci G., Caligiuri M.A., Pro- and anti-inflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells, Immunity (2006) 24:575–590.

[46] Ziv G., Treatment of peracute and acute mastitis, Vet. Clin. North Am. Food Anim. Pract. (1992) 8:1–15.