Expression of macrophage CD14 receptor in the course of experimental inflammatory responses induced by lipopolysaccharide and muramyl dipeptide

Z. Sladek[1,2], D. Rysanek[2]

[1]Mendel University of Agriculture and Forestry, Brno, Czech Republic
[2]Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: The aim of this study was to determine whether expression of CD14 on macrophages is regulated differently during initiation and resolution of the inflammatory response caused by CD14-dependent (lipopolysaccharide) and CD14-independent (muramyldipeptide) bacterial signals. In cell suspensions from the site of inflammation we observed two types of macrophages: non-vacuolized ($_{N}$MAC) and vacuolized ($_{V}$MAC) cells. $_{N}$MAC (monocyte-like cells) were dominant during the early stage of the inflammatory response, whilst $_{V}$MAC contained phagocytosed apoptotic neutrophils in various stages of digestion. These latter cells were dominant during resolution (particularly at the last time point of 168 h). Intramammary instillation of muramyldipeptide (MDP) and lipopolysaccharide (LPS) resulted in a significant increase in the total count of CD14+ $_{N}$MAC after 24 h (muramyldipeptide $P < 0.01$ and lipopolysaccharide $P < 0.05$) compared to phosphate buffered saline (PBS). During resolution of the inflammatory response, a gradual decrease in the total count of CD14+ $_{N}$MAC was observed. The difference compared with PBS was significant at 48 h and 72 h after instillation of both bacterial agents (muramyldipeptide: $P < 0.05$; lipopolysaccharide: $P < 0.05$). A lower total count of CD14+ $_{V}$MAC was observed as an effect of MDP and LPS at 24 h after induction ($P < 0.05$), when compared to PBS. During resolution, the total count of CD14+ $_{V}$MAC increased. Differences ($P < 0.01$) were observed at 72 h and 168 h after LPS compared to PBS. We therefore assume that the expression of CD14 on macrophages is not regulated differently during the inflammatory responses caused by CD14-dependent and CD14-independent bacterial signals. On the other hand, the stage of the inflammatory response to MDP and LPS played an important role in the regulation of CD14 expression on macrophages.

Keywords: mammary gland; macrophages; CD14; muramyldipeptide; lipopolysaccharide

The principal component of the outer membrane of Gram-negative bacteria, lipopolysaccharide (LPS), induces the inflammatory response of an organism (Muhvic et al., 2001). Recognition of LPS by immune cells is the first step in the inflammatory response and, therefore, these cells possess some receptors on their surface for this purpose. The membrane glycoprotein CD14 (molecular mass of 55 kDa), is the main receptor for LPS and is expressed predominantly on the surface of monocytes and macrophages (Jiang et al., 1997). Further, purified lipolic or polysaccharide components such as peptidoglycan (PGN) from Gram-positive bacteria, lipoarabinomannan, or phaspholipid, interact with CD14, suggesting that CD14 acts not only as a surface receptor for LPS but might also function as a polyspecific receptor with broad recognition properties (Pugin et al., 1994).

The next step in the inflammatory response is the binding of LPS to CD14 and activation of macrophages. LPS needs a LPS binding protein (LBP), which catalyzes the binding of LPS to CD14 (Tapping and Tobias, 1997). The binding of LPS-LBP to CD14 is a prerequisite for cell activation

Supported by the Ministry of Agriculture of the Czech Republic (Grant No. MZE 0002716201).

(Wright et al., 1990). Therefore, the CD14 receptor is a very important molecule to study, particularly during infections caused by bacterial pathogens. Little is presently known about the mechanisms that regulate the expression of CD14 on macrophages at sites of bacterial infections.

Some *in vivo* and *in situ* models appear to show that the expression of CD14 corresponds strongly with LPS (CD14-dependent signal) and/or to the stage of the inflammatory response. For example, LPS increases the expression of CD14 on macrophages during the initial stage of the inflammatory response, as was demonstrated *in vivo* during lung injury or endotoxemia (Yamamoto et al., 1999; Jiang et al., 2003) and acute hepatic injury (Jiang et al., 2001; Xie et al., 2002).

Further, an increased expression has been noted in the resolution stage of LPS induced inflammation of the mammary gland by Sladek et al. (2002). In contrast, a reduced expression of CD14 on macrophages was observed in the early stages of an inflammatory response induced by LPS by Paape et al. (1996). In addition, CD14 positive macrophages represent the large, vacuolized cells with phagocytosed apoptotic neutrophils. This is not surprising, because CD14 serves as a receptor involved in the recognition and phagocytosis of apoptotic cells (Devitt et al., 1998; Gregory, 2000). CD14-dependent clearance of apoptotic cells by macrophages has been demonstrated in both human and murine systems (Devitt et al., 1998; Fadok et al., 1998; Moffat et al., 1999; Schlegel et al., 1999).

It appears that the resolution of an inflammatory response plays a more important role in the regulation of CD14 expression on macrophages *in situ* than the more dangerous CD14-dependent signals from invading pathogens. This suggests that similar dynamics may occur during CD14 expression on macrophages during an inflammatory response caused by the Gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus uberis* (Sladek and Rysanek, 2006).

CD14-dependent signals, however, are not the only means by which inflammatory responses may be induced by bacteria. For example, the peptidoglycan by product muramyl dipeptide (MDP) is a key element in the immune response to Gram-positive bacteria which lack LPS (Tavares et al., 2005). In contrast to LPS, the response to MDP is CD14-independent, and is recognized by nucleotide-binding oligomerization domain 2 (NOD2) but not "Toll-like" receptors such as LPS (Girardin et al., 2003). In this case, we may hypothesize no effect of MDP on expression of CD14 on macrophages. Wang et al. (2001), however, reported a 2-fold increase in surface expression of CD14 on monocytes to MDP, and suggested that MDP primes leukocytes for LPS-induced release of proinflammatory cytokines. Due to these contradictory results, it is difficult to say whether the expression of CD14 on macrophages is regulated during initiation and resolution of the inflammatory response caused by the CD14-independent bacterial signal in the same manner as LPS. Moreover, no relevant data relating to this question could be found in the available literature.

The aim of this study, therefore, was to determine if the expression of CD14 on macrophages is regulated differently during the initiation and resolution of an inflammatory response caused by CD14-dependent and CD14-independent bacterial signals. For this purpose, we used an experimentally induced inflammatory response by lipopolysaccharide (LPS) and muramyldipeptide (MDP).

MATERIAL AND METHODS

Animals

The experiments were carried out on forty mammary glands of ten virgin, clinically healthy Holstein × Bohemian Pied crossbred heifers aged 15 to 20 months. The heifers were housed in an experimental tie-stall barn and fed a standard ration consisting of hay and concentrates with mineral supplements. The experimental tie-stall used in this study is certified and animal care conformed to good care practice protocols. All heifers were free of intramammary infections, as demonstrated through bacteriological examination of mammary lavages.

Experimental design

Before the experimental procedure with MDP and LPS, the mammary glands were treated with phosphate buffered saline (PBS). All four mammary gland sinuses of each heifer were rinsed stepwise with PBS to obtain a cell suspension using the following procedure. The first cell sample was obtained by lavage of the left-front quarter 24 h after administration of PBS. The remaining quarters were rinsed stepwise at three 24-hour intervals and one 96-hour interval in the following order:

left-rear (48 h) → right-front (72 h) → right-rear (at 168 h). These PBS-treated mammary glands were set as a control for the MDP and LPS. Five heifers were then used for MDP instillation and the remaining five heifers for LPS. Subsequent lavages of the mammary gland lumens were obtained in the same manner as described. The total somatic cell count (SCC) was assessed by the fluoro-opto-electronic method. The differential leukocyte count and CD14 positive cells in lavages were assessed by flow cytometry (FCM). All ten heifers were also used as blood cell donors.

Induction of acute mammary gland inflammatory response

Modified urethral catheters (AC5306CH06, Porges S.A., France) were inserted into the teat canal following thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mammary quarter was injected with 10 ml of PBS (0.01M, pH 7.4; NaCL 0.138M; KCL 0.0027M, Sigma, St. Louis, MO, USA; prepared with apyrogenic water) and 1 ml of each lavage was immediately collected back through the catheter directly to the syringe and subsequently used for microbiological examination.

After one month we used an intramammmary instillation 500 μg of a synthetic muramyl dipeptide analogue (nor MurANc-L-Abu-D-IzoGln, supplied by the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic, Prague) dissolved in 10 ml PBS in five heifers (Sladek and Rysanek, 2000; Rysanek et al., 2001) and 5 μg of lipopolysaccharide (LPS from *Escherichia coli* Serotype 0128:B12, Sigma, St. Louis, MO, USA) in 10 ml of PBS for the other five heifers (Wardley et al., 1976).

Blood sampling

Blood was collected in a sterile container with Heparin (Leciva a.s., Dolni Mecholupy, Czech Republic): 1 000 IU/ml in PBS, from the external jugular vein using venipuncture.

Bacteriological examination

Bacteriological examination of all the lavages was performed through culture on blood agar plates (5% washed ram erythrocytes) with aerobic incubation at 37°C for 24 h before every experimental procedure.

Processing of cells

Total mammary SCC were determined using the Fossomatic 90 apparatus (Foss Electric, Denmark) and the procedure recommended in the European Standard (2006). The trypan blue dye exclusion test demonstrated more than 95% cell viability in the fresh cell population by an enumeration of at least 200 cells. The cell suspensions were centrifuged at 4°C and 200 × g for 10 min. One ml of supernatant was retained for resuspension of the pellet. The clearance of neutrophils by macrophages was assessed by staining for myeloperoxoidase (MPO) as described by Henson et al. (1978).

Flow cytometry

Percentages of individual cell types (i.e. granulocytes, macrophages and lymphocytes) were read from forward scatter versus side scatter dot plots as we described previously in Sladek et al. (2002).

An indirect staining technique was used for detection of CD14

Mouse anti-ovine CD14 (VPM65, Serotec, Oxford, UK) diluted 1 : 20 and fluorescein isothiocyanate-labeled swine anti-mouse immunoglobulin (SwM-FITC, Sevac, Prague, Czech Republic), diluted 1 : 500 were used as the primary and secondary antibodies, respectively (Sladek et al., 2002). Negative control samples were stained with the secondary antibody only. For analysis we used the FACS Calibur flow cytometer (FACS) and CELLQuest™ software (Becton Dickinson, Mountain View, CA, USA).

Effect of MDP on CD14 expression *in vitro*

The cell suspension collected 24 h after instillation of PBS was used for *in vitro* cultivation. The cells were adjusted (5×10^6 cells/mL) in an RPMI 1640 (Sigma, MO, USA) and were inserted into microplates (6 × 4 Costar Ultraplates, CA, USA). The cells were incubated for 30, 60 and 120 min

at 37°C, with either MDP or PBS (as a control), in the same final concentration (50 μg/ml) as used for intramammary instillation (see "Induction of acute mammary gland inflammatory response"). Following each incubation period, the cells were analyzed using flow cytometry.

Statistical analysis

The results were subjected to multifactorial analysis of variance (ANOVA) for determination of significant sources of variability. Significance in the differences between means for inflammatory cell numbers, neutrophil and macrophage proportions, and percentage of CD14+ macrophages in response to PBS, MDP and LPS instillations were determined using Scheffe's method. STAT Plus software (Matouskova et al., 1992) was used throughout.

RESULTS

Inflammatory response of mammary glands to PBS, MDP and LPS

Intramammary instillation using PBS, MDP or LPS induced a local inflammatory response from the mammary gland characterized by significant changes in both total and differential inflammatory cell counts. The total cell count increased significantly during the initial stage of the inflammatory response (after 24 h), and the response to MDP and LPS was significantly stronger than that to PBS ($P < 0.01$). At 48 h, the increase was followed by a decrease in total cell counts that was observed up to 168 h after instillation (Figure 1).

Neutrophils were the predominant cell type in the population obtained 24 h after instillation (PBS versus LPS: $P < 0.05$). Between 24 and 72 h after initiation of the inflammatory response neutrophils gradually declined (Figure 2). Over the same period, macrophages increased such that, at 168 h, macrophages made up around 65–79% of the exudates' cells (Figure 3). The decrease in the cell count and proportion of neutrophils, along with the increased proportion of macrophages, characterized the resolution stage of the inflammatory response. Clearance of neutrophils was more pronounced and more rapid after the instillation of PBS than after the MDP or LPS. Simultaneously, the proportion of MPO+ macrophages reflected the rate of resolution. For PBS, resolution had already started after 24 h, and terminated at 168 h. For both the MDP- and LPS-induced inflammatory responses, resolution peaked at 48 h and 72 h, respectively (Figure 4).

Careful examination of the cell population by light microscopy revealed that the population of macrophages included two cell types: non-vacuolized ($_N$MAC) and vacuolized ($_V$MAC) cells (for details see Sladek and Rysanek, 1999). $_N$MAC (monocyte-like cells) were dominant during the early stage of the inflammatory response. $_V$MAC predominantly contained phagocytosed apoptotic neutrophils in various stages of digestion. These cells were dominant during resolution, and particularly so at 168 h (Figure 3).

Evaluation of CD14+ macrophages

We observed 52.9 ± 8.8% CD14+ monocytes in the blood, and 57.5 ± 12.6% CD14+ $_N$MAC and

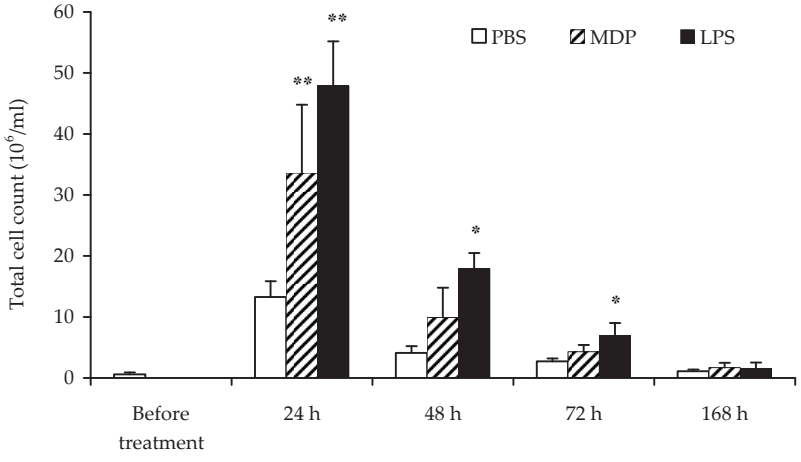

Figure 1. Total number of cells (mean ± SD) in mammary lavages collected before and at 24, 48, 72, and 168 h after intramammary instillation of PBS, MDP or LPS. Significance between instillation differences in total number of cells is indicated with asterisks (**$P < 0.01$, *$P < 0.05$)

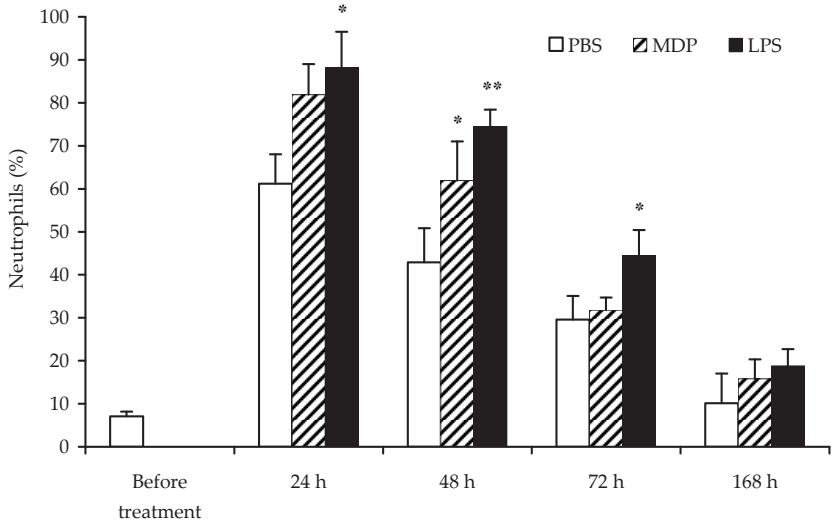

Figure 2. Proportion of neutrophils (mean ± SD) in mammary lavages collected before and at 24, 48, 72, and 168 h after intramammary instillation of PBS, MDP or LPS. Significance between instillation differences in proportion of the neutrophils is indicated with asterisks (**$P < 0.01$, *$P < 0.05$)

67.9 ± 9.7% CD14+ $_{V}$MAC in untreated mammary glands before instillation. Following the intramammary application of PBS, MDP and LPS, and during the initiation and resolution of the inflammatory response to these agents, total count and percentage of CD14+ macrophages detected changed.

Non-vacuolized macrophages ($_{N}$MAC). The initial phase of the inflammatory response to PBS, MDP and LPS resulted in an increase in the total count of CD14+ $_{N}$MAC (Figure 5a). However, whereas a peak in the CD14+ $_{N}$MAC was observed for PBS and MDP at 24 h, the peak was at 48 h

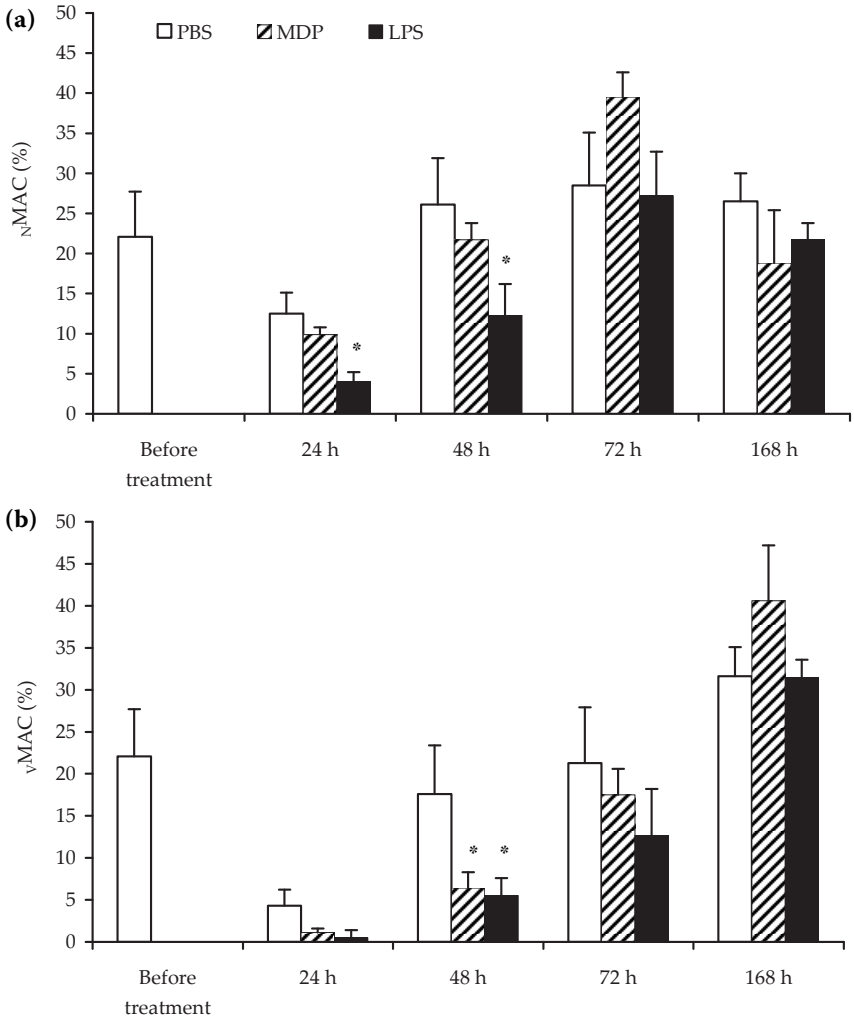

Figure 3. Proportion of (a) non-vacuolized macrophages and (b) vacuolized macrophages in mammary lavages collected before and at 24, 48, 72, and 168 h after intramammary instillation of PBS or MDP or LPS (mean ± S.D.). Significance between instillation differences in proportion of these cells is indicated with asterisks (*$P < 0.05$)

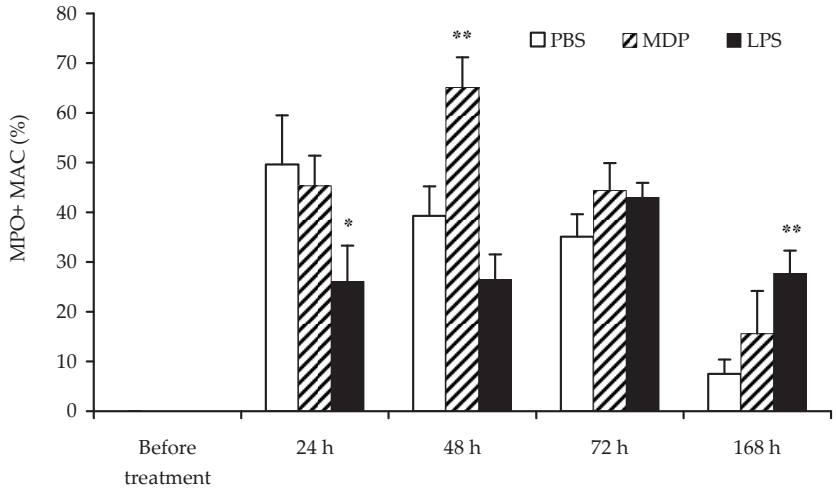

Figure 4. Proportion of MPO+ macrophages (mean ± SD) in mammary lavages collected before and at 24, 48, 72, and 168 h after intramammary instillation of PBS, MDP or LPS. Significance between instillation differences is indicated with asterisks (**$P < 0.01$, *$P < 0.05$)

for LPS. A statistically significant lower count of CD14+ $_N$MAC was measured for PBS than for MDP ($P < 0.01 \rightarrow$ 24 h; $P < 0.05 \rightarrow$ 48 h) and LPS ($P < 0.05 \rightarrow$ 24–72 h) (Figure 5a).

Intramammary application of MDP and LPS, therefore, resulted in a statistically significant increase in the proportion of CD14+ $_N$MAC during the initial stage of the inflammatory response in

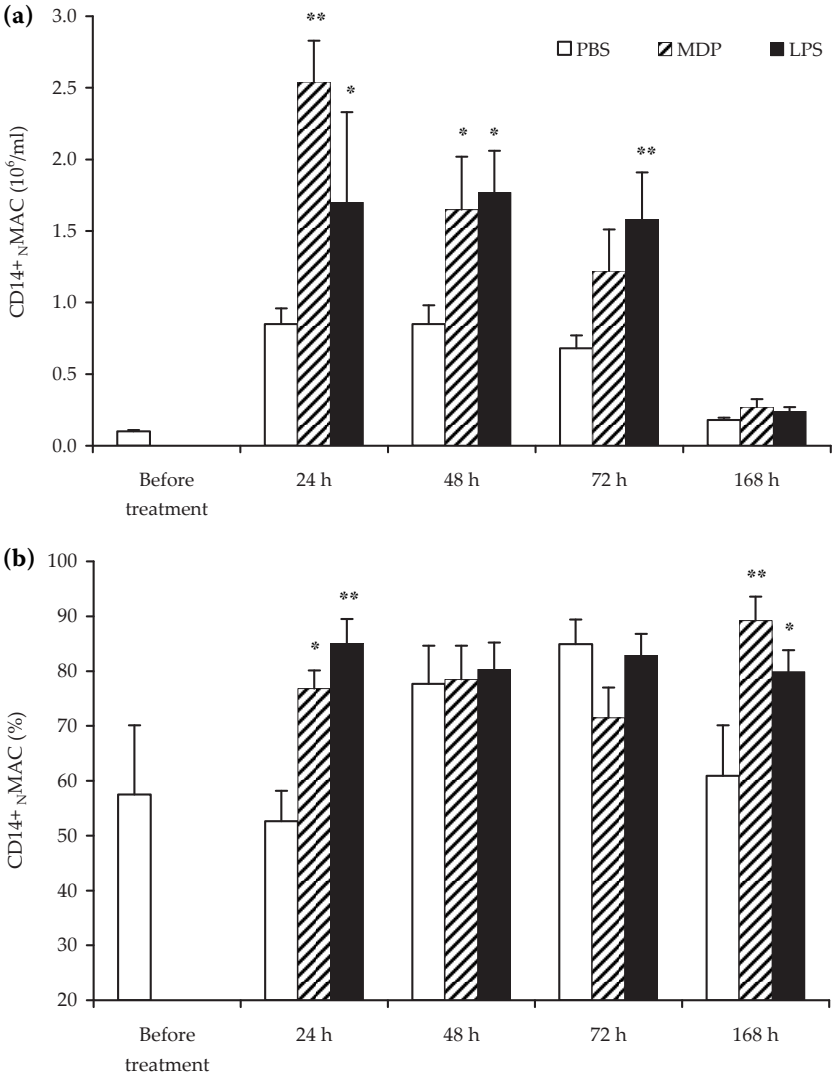

Figure 5. (a) Total number of CD14+ $_N$MAC and (b) relative proportion of CD14+ $_N$MAC (mean ± SD) in mammary lavages collected before and at 24, 48, 72, and 168 h after intramammary instillation of PBS, MDP or LPS. Significance between instillation differences is indicated with asterisks (**$P < 0.01$, *$P < 0.05$)

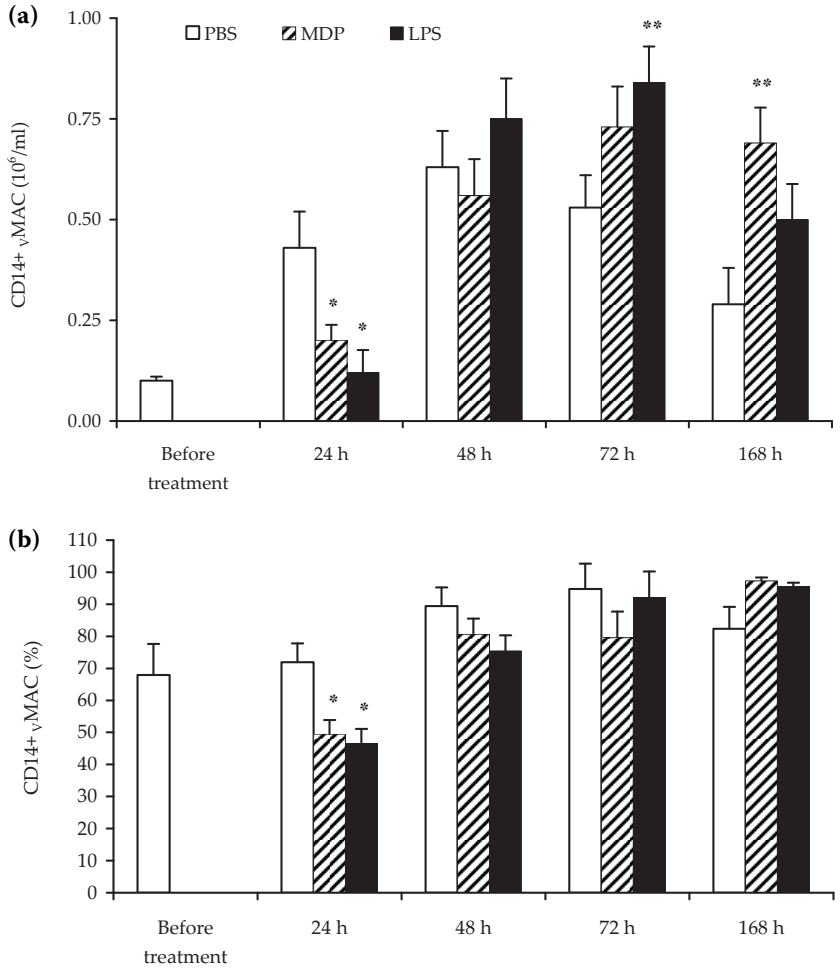

Figure 6. (a) Total number of CD14+ $_V$MAC and (b) relative proportion of CD14+ $_V$MAC (mean ± SD) in mammary lavages collected before and at 24, 48, 72, and 168 h after intramammary instillation of PBS, MDP or LPS. Significance between instillation differences is indicated with asterisks (**$P < 0.01$, *$P < 0.05$)

comparison with PBS ($P < 0.05$ in MDP and $P < 0.01$ in LPS). The proportion of CD14+ $_N$MAC for PBS, however, in contrast to that for MDP and LPS, increased over the 48 h and 72 h time periods and decreased at 168 h (Figure 5b).

Vacuolized macrophages ($_V$MAC). A similar effect was observed for both MDP and LPS for both the total and differential counts of CD14+ cells in the population of $_V$MAC in the initial phase of the inflammatory response. This was characterized by

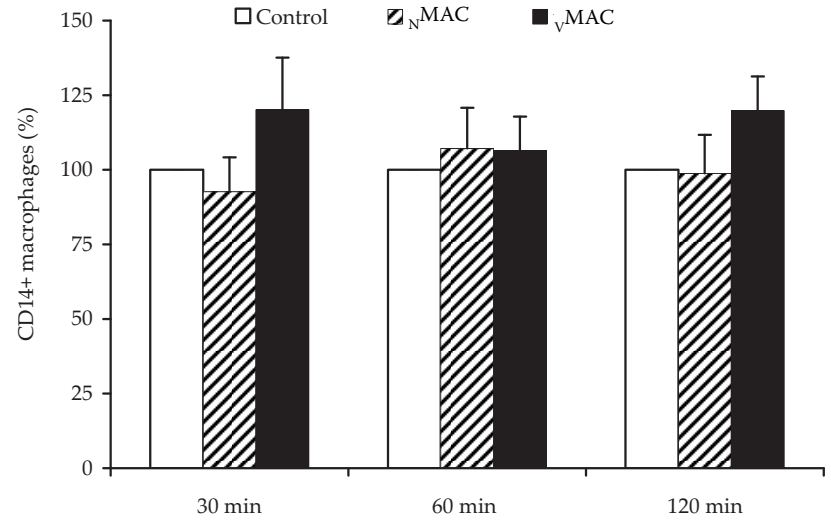

Figure 7. Relative proportion of CD14+ $_N$MAC and CD14+ $_V$MAC after 30, 60 and 120 min cultivation *in vitro* with MDP and without (control)

a statistically significant decrease in total count and proportion of CD14+ $_{V}$MAC following instillation with MDP and LPS (Figure 6). During resolution, the total count of these cells increased for MDP and LPS up to 72 h after instillation in contrast to PBS, which only increased up to 48 h (Figure 6a). A similar situation was observed in the proportion of CD14+ $_{V}$MAC, whereby these cells increased up to 168 h after instillation with MDP and LPS, but decreased for PBS after 72 h (Figure 6b).

No statistically significant differences in total and differential CD14+ macrophage counts were observed between MDP and LPS throughout the experimental period.

Effect of MDP on CD14 expression *in vitro*

As mentioned above, after instillation with MDP we observed an increased proportion of CD14+ $_{N}$MAC and a decreased proportion of CD14+ $_{V}$MAC, in comparison to PBS. We therefore examined the possible effect of MDP on CD14 expression *in vitro*.

No statistically significant differences in the proportions of CD14+ $_{N}$MAC and CD14+ $_{V}$MAC were observed after 30, 60 and 120 min *in vitro* incubation with either MDP or PBS (control), although unexpectedly higher proportions of CD14+ cells were detected for $_{V}$MAC than for $_{N}$MAC (Figure 7).

DISCUSSION

The aim of this study was to investigate whether expression of CD14 on macrophages is differently regulated during initiation and resolution of inflammatory response caused by CD14-dependent and CD14-independent bacterial signal. To our knowledge, this is the first report on a comparison of CD14 expression on two types of macrophages throughout the period of inflammatory response induced by signals with different mechanisms of cell activation.

For this purpose we used a model for bacterial infection, induced using intramammary instillation of LPS and MDP. LPS, as a CD14-dependent signal, is believed to be the most important marker for Gram-negative bacteria and imitates infections of the mammary gland by *E. coli* (Paape et al., 1996; Mehrzad et al., 2001). The peptidoglycan by-product MDP, on the other hand, representing a CD14-independent signal, is a key element in the immune response to Gram-positive bacteria, as it serves as a salient stimulus from these bacteria (Dinarelo et al., 1978). As a control, we used PBS, non-bacterial, CD14-independent signal.

The intramammary instillation of PBS, MDP and LPS resulted in an inflammatory response with distinguishable initial and resolution stages. We observed a massive influx of neutrophils from blood into the mammary glands during the initial stage of the inflammatory response. The influx of neutrophils culminated at 24 h, and resolution occurred during 48–168 h period as neutrophils underwent apoptosis and were subsequently phagocytosed by macrophages (Sladek and Rysanek, 2000, 2001). Inflammatory macrophages migrate from the blood through the surrounding tissues into the cavity of the mammary gland and possess monocyte-like morphology. During resolution, these cells are vacuolized due to their scavenger function. These results were consistent with our previous findings in the experimentally induced inflammatory response of the mammary gland to various bacterial agents (Sladek and Rysanek, 2000, 2001, Sladek et al., 2005, 2006).

Instillation of mammary glands with MDP and LPS resulted in a statistically significant increase in the total count and the proportion of CD14+ $_{N}$MAC in comparison to PBS. This increase was caused by activation of newly migrated macrophages from the blood induced by bacterial components (Paape et al., 1996; Sladek et al., 2002). LPS is an important bacterial immunomodulator and it is known that it increases the expression of CD14 on macrophages and the CD14 gene expression (Pure and Cuff, 2001). The up-regulating effect of LPS on macrophages has previously been described by Landmann et al. (1991) and Chen et al. (1992). In our previous study (Sladek et al., 2002) we observed this up-regulating effect of LPS on bovine mammary gland $_{N}$MAC during the initial stage of the inflammatory response.

Whereas the effect of LPS on cells of the immune system is relatively well known, little is known about MDP. It has been observed that this "smallest immunologically active structure of the Gram-positive bacterial cell wall" (Masek, 1986) has the effect of expression of CD14. Wang et al. (2001) reported a 2-fold increase in surface expression of CD14 on monocytes and suggested that both PGN and MDP prime leukocytes for LPS induced the release of proinflammatory cytokines. This syn-

ergy between MDP and LPS may contribute to the pathogenesis in sepsis caused by mixed bacterial infections. In this study, we observed a higher total count and proportion of CD14+ $_N$MAC after MDP than after PBS. This was unexpected inasmuch as the immune response to MDP is CD14 independent in contrast to that of LPS (Girardin et al., 2003). It may be that the various molecules recognizing CD14, including MDP, differ greatly in chemical structure but share a glycosyl region which may be involved in the interaction with CD14 (Weidemann et al., 1997). Indeed, it is already known that MDP can also block PGN binding to CD14, suggesting that CD14 represents a cellular MDP receptor (Weidemann et al., 1997).

In contrast to the results mentioned above, which were obtained on blood monocytes, when we incubated $_N$MAC with MDP *in vitro*, MDP did not increase the expression of CD14 in these cells. The reason for this is unknown. We can hypothesize that the fine specificity of CD14 alters during the maturation of monocytes to macrophages, however, this question is not yet fully resolved.

In contrast to $_N$MAC, it was observed that instillation of mammary glands by MDP and LPS resulted in a statistically significant decrease in both the total count and the proportion of CD14+ $_V$MAC, in comparison to PBS. This would appear to represent endotoxine toleration. It has been demonstrated, however, that a down-regulation in the expression of the CD14 LPS receptor is not the mechanism by which LPS tolerance is induced, as the expression of the CD14 receptor remained almost unchanged in observation of peritoneal macrophages of rabbits adapted to LPS (Mathison et al., 1990). There is no doubt that the down-regulation of receptors could be important for the prevention of an overwhelming release of proinflammatory cytokines, thus preventing the development of septic shock (Dobrovolskaia and Vogel, 2002). This is because, monocytes/macrophages release a spectrum of proinflammatory cytokines in response to LPS/LBP complexes, including tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8, to initiate host defense (Martin, 2000). Similarly, MDP is capable of stimulating NF-κB-dependent gene transcription and pro-inflammatory cytokine production (Windheim et al., 2007).

A father explanation may be that CD14 is shed from the membrane into the extracellular space during the interaction of macrophages with bacteria and their components. It is known that LPS induces shedding of CD14 into the extracellular space (Sugawara et al., 2000) and this soluble form of CD14 is derived from the shedding of membrane CD14 from CD14 bearing cells (Tapping and Tobias, 1997). Increased levels of the soluble form of CD14 were observed during mastitis caused by *E. coli* (Paape et al., 1996; Bannerman et al., 2004a; Vangroenweghe et al., 2004), and, surprisingly, by *S. aureus* (Bannerman et al., 2004a) and *S. uberis* (Bannerman et al., 2004b). Moreover, we observed the down-regulation of CD14 expression on $_V$MAC during experimental mastitis caused by *S. aureus* and *S. uberis* (Sladek and Rysanek, 2006).

When we incubated macrophages with MDP *in vitro*, however, CD14 expression was increased in $_V$MAC, though the differences were statistically insignificant when compared with PBS. Why is the expression of CD14 in $_V$MAC *in vitro* higher than *in situ*? We suggested that the reason why expression of CD14 in $_V$MAC *in vitro* is higher then that *in situ* is that it is related to the scavenger function of macrophages, which is associated with a change in their morphology. For *in vitro* assay, we used macrophages obtained 24 h after intramammary instillation of PBS. As we have demonstrated in our previous studies (Sladek and Rysanek 2000, 2001; Sladek et al., 2005, 2006), $_N$MAC are involved in the clearance of apoptotic neutrophils, particularly during the period 24 to 48 h after induction of the inflammatory response. Phagocytosis of the apoptotic neutrophils results in vacuolization of $_N$MAC and their translocation into the region of $_V$MAC in dot-plots analyzed using FACS (Sladek et al., 2002). Moreover, the phagocytosing macrophages are CD14 positive cells, as CD14 is an important receptor for the uptake of apoptotic neutrophils (Hart et al., 1997; Devitt et al., 1998). We suggest, therefore, that the increase in the proportion of CD14+ $_V$MAC is not the result of a direct MDP effect but an outgrowth of the clearing process.

To summarize, in this work we observed no significant differences in total counts and in the proportions of CD14+ $_N$MAC and CD14+ $_V$MAC during an inflammatory response to LPS and MDP. We therefore suggest that the expression of CD14 on macrophages is not regulated during the inflammatory response caused by CD14-dependent and CD14-independent bacterial signals. On the other hand, the stage of the inflammatory response to MDP and LPS does play an important role in the regulation of CD14 expression on macrophages.

REFERENCES

Bannerman D.D., Paape M.J., Lee J.W., Zhao X., Hope J.C., Rainard P. (2004a): *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clinical Diagnose Laboratory Immunology, 11, 463–472.

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

Chen T.Y., Lei M.G., Suzuki T., Morrison D.C. (1992): Lipopolysaccharide receptors and signal transduction pathways in mononuclear phagocytes. *Current Topics in Microbiology and Immunology, 181,* 169–188.

Devitt A., Moffatt O.D., Raykundalia C., Capra J.D., Simmons D.L., Gregory C.D. (1998): Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature, 392, 505–509.

Dinarello C.A., Elin R.J., Chedid L., Wolff S.M. (1978): The pyrogenicity of the synthetic adjuvant muramyl dipeptide and two structural analogues. Journal of Infectious Diseases, 138, 760–767.

Dobrovolskaia M.A., Vogel S.N. (2002): Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes and Infection, 42, 903–914.

European Standard (EN ISO 13366-2) (2006): Mil – Enumeration of Somatic Cell. Part 2: Guidance on the Operation of Fluoro-opto-electronic Counters.

Fadok V.A., Warner M.L., Bratton D.L., Henson P.M. (1998): CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha(v)beta(3)). Journal of Immunology, 161, 6250–6257.

Girardin S.E., Boneca I.G., Viala J., Chamaillard M., Labigne A., Thomas G., Philpott D.J., Sansonetti P.J. (2003): Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. Journal of Biology and Chemistry, 14, 8869–8872.

Gregory C.D. (2000): CD14-dependent clearance of apoptotic cells: relevance to the immune system. Current Opinion in Immunology, 12, 27–34.

Hart S.P., Dougherty G.J., Haslett C., Dransfield I. (1997): CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages. Journal of Immunology, 159, 919–925.

Henson P.M., Zanolari B., Schwartzman N.A., Hong S.R. (1978): Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. Journal of Immunology, 121, 851–855.

Jiang J., Diao Y., Tian K. (1997): Increased sensitivity to endotoxin and its molecular mechanism after hemorrhagic shock. Zhonghua Yi Xue Za Zhi, 77, 282–285.

Jiang J., Xie G., Chen Y., Liu D., Qiu J., Zhou J., Zhu P., Wang Z. (2001): Intra-hepatic expression of scavenger receptor and CD14 and their relationship with local inflammatory responses in endotoxemia in mice. Shock, 16, 75–80.

Jiang J.X., Chen Y.H., Xie G.Q., Ji S.H., Liu D.W., Qiu J., Zhu P.F., Wang Z.G. (2003): Intrapulmonary expression of scavenger receptor and CD14 and their relation to local inflammatory responses to endotoxemia in mice. World Journal of Surgery, 27, 1–9.

Landmann R., Ludwig C., Obrist R., Obrecht J.P. (1991): Effect of cytokines and lipopolysaccharide on CD14 antigen expression in human monocytes and macrophages. Journal of Cellular Biochemistry, 47, 317–329.

Martin T.R. (2000): Recognition of bacterial endotoxin in the lungs. American Journal of Respiratory Cell and Molecular Biology, 23, 128–132.

Masek K. (1986): Immunopharmacology of muramyl peptides. Federation Proceedings, 45, 2549–2551.

Mathison J.C., Wolfson E., Steinemann S., Tobias P., Ulevitch R. (1990): Lipopolysaccharide (LPS) recognition in macrophages: participation of LPS-binding protein and CD14 in LPS-induced adaptation in rabbit peritoneal exudate macrophages. Journal of Clinical Investigation, 92, 2053–2059.

Matouskova O., Chalupa J., Cigler M., Hruska K. (1990): STAT Plus – Manual (in Czech). 1st ed. Veterinary Research Institute, Brno. 168 pp.

Mehrzad J., Dosogne H., Meyer E., Burvenich C. (2001): Local and systemic effects of endotoxin mastitis on the chemiluminescence of milk and blood neutrophils in dairy cows. Veterinary Research, 32, 131–144.

Moffatt O.D., Devitt A., Bell E.D., Simmons D.L., Gregory C.D. (1999): Macrophage recognition of ICAM-3 on apoptotic leukocytes. Journal of Immunology, 162, 6800–6810.

Muhvic D., El-Samalouti V., Flad H.D., Radosevic-Stasic B., Rukavina D. (2001): The involvement of CD14 in the activation of human monocytes by peptidoglycan monomers. Mediators in Inflammation, 10, 155–162.

Paape M.J., Lilius E.M., Wiitanen P.A., Kontio M.P., Miller R.H. (1996): Intramammary defense against infections induced by *Escherichia coli* in cows. American Journal of Veterinary Research, 57, 477–482.

Pugin J., Heumann I.D., Tomasz A., Kravchenko V.V., Akamatsu Y., Nishijima M., Glauser M.P., Tobias P.S., Ulevitch R.J. (1994): CD14 is a pattern recognition receptor. Immunity, 1, 509–516.

Pure E., Cuff C.A. (2001): A crucial role for CD44 in inflammation. Trends in Molecular Medicine, 7, 213–221.

Rysanek D., Babak V., Sladek Z., Toman M. (2001): Variations among unbred heifers in the activities of polymorphonuclear leucocytes from the mammary gland and blood. Journal of Veterinary Medicine B, 48, 31–41.

Schlegel R.A., Krahling S., Callahan M.K., Williamson P. (1999): CD14 is a component of multiple recognition systems used by macrophages to phagocytose apoptotic lymphocytes. Cell Death and Differentiation, 6, 583–592.

Sladek Z., Rysanek D. (1999): Ultrastructure of phagocytes from mammary glands of non-pregnant heifers. Anatomia, Histologia, Embryologia, 28, 291–297.

Sladek Z., Rysanek D. (2000): Apoptosis of polymorphonuclear leukocytes of the juvenile bovine mammary gland during induced influx. Veterinary Research, 31, 553–563.

Sladek Z., Rysanek D. (2001): Neutrophil apoptosis during the resolution of bovine mammary gland injury. Research in Veterinary Science, 70, 41–46.

Sladek Z., Rysanek D. (2006): The role of CD14 during resolution of experimentally induced *Staphylococcus aureus* and *Streptococcus uberis* mastitis. Comparative Immunology, Microbiology and Infectious Diseases, 29, 243–262.

Sladek Z., Rysanek D., Faldyna M. (2002): Activation of phagocytes during initiation and resolution of mammary gland injury induced by lipopolysaccharide in heifers. Veterinary Research, 33, 191–204.

Sladek Z., Rysanek D., Ryznarova H., Faldyna M. (2005): Neutrophil apoptosis during experimentally induced *Staphylococcus aureus* mastitis. Veterinary Research, 36, 629–643.

Sladek Z., Rysanek D., Ryznarova H., Faldyna M. (2006): The role neutrophil apoptosis during experimentally induced *Streptococcus uberis* mastitis. Veterinarni Medicina, 51, 437–447.

Sugawara S., Nemoto E., Tada H., Miyake K., Imamura T., Takada H. (2000): Proteolysis of human monocyte CD14 by cysteine proteinases (gingipains) from *Porphyromonas gingivalis* leading to lipopolysaccharide hyporesponsiveness. Journal of Immunology, 165, 411–418.

Tapping R.I., Tobias P.S. (1997): Cellular binding of soluble CD14 requires lipopolysaccharide (LPS) and LPS-binding protein. Journal of Cellular Biochemistry, 272, 23157–23164.

Tavares E., Maldonado R., Ojeda M.L., Minano F.J. (2005): Circulating inflammatory mediators during start of fever in differential diagnosis of Gram-negative and Gram-positive infections in leukopenic rats. Clinical and Diagnostic Laboratory Immunology, 12, 1085–1093.

Vangroenweghe F., Rainard P., Paape M., Duchateau L., Burvenich C. (2004): Increase of E. coli inoculums doses induces faster innate immune response in primiparous cows. Journal of Dairy Science, 87, 4132–4144.

Wang J.E., Jorgensen P.F., Ellingsen E.A., Almiof M., Thiemermann C., Foster S.J., Aasen A.O., Solberg R. (2001): Peptidoglycan primes for LPS-induced release of proinflammatory cytokines in whole human blood. Shock, 16, 178–182.

Wardley R.C., Rouse B.T., Babiuk L.A. (1976): The mammary gland of the ox: a convenient source for the repeated collection of neutrophils and macrophages. Journal of the Reticuloendothelial Society, 19, 29–36.

Weidemann B., Schletter J., Dziarski R., Kusumoto S., Stelter F., Rietschel E.T., Flad H.D., Ulmer A.J. (1997): Specific binding of soluble peptidoglycan and muramyldipeptide to CD14 on human monocytes. Infection and Immunity, 65, 858–864.

Windheim M., Lang C., Peggie M., Plater L.A., Cohen P. (2007): Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. Biochemistry Journal, 404, 179–190.

Wright S.D., Ramos R.A., Tobias P.S., Ulevitch R.J., Mathison J.C. (1990): CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science, 21, 1431–1433.

Xie G.Q., Jiang J.X., Chen Y.H., Liu D.W., Zhu P.F., Wang Z.G. (2002): Induction of acute hepatic injury by endotoxin in mice. Hepatobiliary and Pancreatic Diseases International, 1, 558–564.

Yamamoto T., Ebe Y., Hasegawa G., Kataoka M., Yamamoto S., Naito M. (1999): Expression of scavenger receptor class A and CD14 in lipopolysaccharide-induced lung injury. International Journal of Surgical Patology, 49, 983–992.

Received: 2008–02–07
Accepted after corrections: 2008–07–16

Corresponding Author:

Doc. MVDr. Zbysek Sladek, PhD., Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
Tel. +420 533 331 501, e-mail: sladekz@seznam.cz