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Original article

Activation of phagocytes during initiation and resolution of mammary gland injury induced by lipopolysaccharide in heifers

Zbyšek SLÁDEK[a]*, Dušan RYŠÁNEK[b], Martin FALDYNA[b]

[a] Department of Morphology, Physiology and Veterinary Sciences, Mendel University of Agriculture and Forestry, Zemedelská 1, 613 00 Brno, Czech Republic
[b] Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic

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Abstract – The object of the study was the comparative assessment of phagocyte activation during initiation and resolution of mammary gland injury induced by lipopolysaccharide (LPS) or buffered salt solution (PBS) on the basis of the CD14 receptor positivity. The experiments were carried out in 15 clinically normal Holstein × Bohemian Red Pied crossbred heifers, aged 14 to 18 months. Non-inflammatory and inflammatory mammary gland injury were induced by intramammary administration of PBS (10 mL) and LPS (10 mL, 1 μg/mL), respectively. Samples of the cell populations were obtained by mammary lavages at 24 h intervals. Flow cytometry was used to determine the CD14+ neutrophils, monocytes, and macrophages. The percentage of CD14+ neutrophils was only 1.2% and 1.3% 24 h after the treatment with PBS and LPS, respectively. The resolution was accompanied by an increase in proportion of CD14+ neutrophils. The proportion of CD14+ neutrophils returned to initial values in the PBS-treated, but not in the LPS-treated mammary glands till 96 h. Percentage of CD14+ monocytes increased after 24 h and the effect was more pronounced in the LPS-treated than in the PBS treated mammary glands ($P < 0.05$). The percentage of CD14+ macrophages decreased highly significantly at 24 h in the LPS-treated, but not in the PBS-treated mammary glands ($P < 0.01$). The resolution of mammary gland injury (48 to 96 h) was characterised by an increase in CD14+ macrophages proportion, which was greater in the LPS-treated than PBS-treated mammary glands ($P < 0.01$). The activation of macrophages during resolution of mammary gland injury can be interpreted as an important mechanism of restitution.

heifers / mammary gland injury / lipopolysaccharide / phagocyte / CD14

*Correspondence and reprints
Tel.: (42) 0 5 45 13 31 51; fax: (42) 0 5 45 21 11 28; e-mail: sladekz@seznam.cz

Résumé – **Activation des phagocytes au cours de l'initiation et de la résolution de lésion de la glande mammaire induite par des lipopolysaccharides chez des génisses.** L'objet de la présente étude est l'analyse de l'activation des phagocytes au cours de l'initiation et de la résolution de lésion de la glande mammaire induite par des lipopolysaccharides (LPS), ou par la solution physiologique (PBS), et cela sur la base de la positivité du CD14 récepteur. L'essai fut réalisé sur 15 génisses, cliniquement en bonne santé, issues du croisement des races Holstein × Pie rouge tchèque, âgées de 14 à 18 mois. Des lésions non-inflammatoires et inflammatoires de la glande mammaire ont été induites par l'administration intra-mammaire de PBS (10 mL) et LPS (10 mL, 1 μg/mL), respectivement. Les échantillons de populations cellulaires ont été obtenus par lavage des glandes mammaires, à des intervalles de 24 heures. On a utilisé la cytométrie de flux pour détecter les neutrophiles, les monocytes et les macrophages CD14+. Pendant l'induction de la lésion de glande mammaire (24 h), on a détecté seulement 1,2 % des neutrophiles CD14+ après traitement par le PBS et 1,3 % après traitement par le LPS. La résolution fut caractérisée par une augmentation de la proportion de neutrophiles CD14+. Jusqu'à 96 heures, après injection de PBS, à la différence de celle de LPS, la proportion de neutrophiles CD14+ a retrouvé les valeurs observées avant l'induction. Après 24 heures, la proportion relative des monocytes CD14+ fut plus élevée suite à l'injection de LPS que suite à celle de PBS ($P < 0.05$). Suite à l'administration de LPS, à la différence de celle de PBS, une baisse très importante du nombre relatif des macrophages CD14+ ($P < 0.01$) se manifesta pendant 24 heures. La résolution de lésion de la glande mammaire (pendant la période de 48 à 96 heures) fut caractérisée par une augmentation des macrophages CD14+ ($P < 0.01$) qui fut plus importante suite à l'administration de LPS que suite à l'administration de PBS. L'activation des macrophages au cours de la résolution de lésion de la glande mammaire, peut être considérée comme un mécanisme de restitution important.

génisse / lésion de la glande mammaire / lipopolysaccharide / phagocyte / CD14

1. INTRODUCTION

Bacterial lipopolysaccharide (LPS) is a strong toxin released from the cell wall of Gram-negative bacteria, which is often used as a pro-inflammatory agent [1, 18]. Infusion of LPS into the bovine mammary gland induces an inflammatory response which is a suitable model for studies of biochemical, pathophysiological and immunological aspects of acute mammary gland inflammation [14, 16, 17, 19, 26]. The initial phase of the LPS-induced inflammatory response is characterised by a massive influx of neutrophils which are highly active phagocytes removing bacterial agents from infected mammary gland tissues. Mononuclear phagocytes (monocytes and macrophages), which are responsible for pro-inflammatory processes are also activated by LPS [2].

The LPS-mediated activation of monocytes, macrophages and neutrophils includes two key components which are the LPS-binding protein (LBP) and the CD14 receptor [25]. Activated cells express on their surfaces CD14 which binds LPS in a complex with LBP. This binding initiates in macrophages the production of pro-inflammatory cytokines, such as tumour necrosis factor- alpha (TNF-α) and interleukin 6 [3]. The CD14 receptor also binds cell wall components of Gram-positive bacteria, fungi, and spirochetes. Such activation provides a nonspecific defence system against both Gram-negative and Gram-positive bacteria, because CD14 is a polyspecific receptor with a manifold recognition potential [13, 26].

Most studies demonstrating the importance of cellular CD14 in the activation of mononuclear phagocytes were carried out using human monocytes or monocytoid cell lines, but papers demonstrating similar interactions in bovine pulmonary macrophages [8, 28], and macrophages and neutrophils in LPS-induced inflammatory responses in

bovine mammary gland [14] have also been published.

Paape et al. [14] observed a pronounced down-regulation of CD14 expression in neutrophils in the initial phase of the LPS-induced inflammatory response in the bovine mammary gland. Thereby, the production of interleukin-1 and TNF-α is inhibited [22]. On the contrary, the concentrations of these cytokines increase in the initial phase of the LPS-induced inflammatory response and decrease during its resolution [17, 22].

Nothing is known on the expression of CD14 in phagocytes during the phase of resolution of mammary gland acute inflammation. This is a very important phase which leads to structural and functional restitution of the damaged tissue [7]. The resolution of LPS-induced mammary gland inflammation is characterised by significant interactions among inflammatory cells which result in the subsidence of influx by apoptosis of neutrophils. Mononuclear phagocytes of the cavity system of the mammary gland participates in the clearance of apoptotic neutrophils by phagocytosis [23, 24]. Mononuclear phagocytes of heifers mammary glands include two morphologically distinct cell types. The major portion in the initial phase of mammary gland injury are small, vacuole-free, blood monocyte-resembling monocytes that have recently migrated from surrounding tissues or the blood. The larger, vacuolised macrophages are resident cells which can be found mostly in intact heifers mammary glands and also during the resolution of mammary gland injury [27].

Hence, the resolution of LPS-induced inflammation is accompanied by a decrease in the level of pro-inflammatory cytokines and intensive functional exploitation of mononuclear phagocytes. It can therefore be expected that, unlike the initial phase, the resolution of LPS-induced inflammation will be associated with an activation of neutrophils and mononuclear phagocytes characterised by an increased of proportion of CD14 positive cells.

The aim of the study was to establish whether resolution of LPS-induced mammary gland injury is accompanied by a change in the proportions of CD14-positive neutrophils, monocytes, and macrophages.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

The experiments were carried out in fifteen clinically normal Holstein × Bohemian Red Pied crossbred heifers aged 14 to 18 months. The heifers were free of intramammary infections, as demonstrated by a bacteriological examination of cell suspensions obtained by mammary lavage. Ten heifers were used as blood cell donors for in vivo studies of CD14 expression and five heifers as mammary gland cell donors for in vivo studies of CD14 expression during the resolution of mammary gland injury induced by intramammary administration of either PBS (non-inflammatory response) or LPS (pro-inflammatory response).

2.2. Induction of acute mammary gland injury

Modified uretheral catheters (AC5306 CH06, Porges S.A., France) were inserted into the teat canal after thorough disinfection of the teat orifice with 70% ethanol. Through the catheter each mammary quarter was injected with 20 mL of PBS, pH 7.4, and immediately lavages were collected back through the catheter directly to the syringe for obtaining control cell population. The lavage was followed by administration of PBS or LPS by the same way. In the first experiment 10 mL of PBS and 10 μg of LPS (LPS of *Escherichia coli* serotype O128: B12, Sigma, St. Louis, Mo., USA) in 10 mL of PBS in the second experiment conducted on the same animals approximately two months

apart. Samples of cell populations were obtained by mammary lavage of the left forequarter done 24 h after the treatment with PBS or LPS and repeated at 24 h intervals from the remaining quarters in the following order: left hindquarter → right forequarter →right hindquarter. The resulting mammary lavages were designated in terms of the before and post-treatment intervals as control (pre-treatment) and 24 h to 96 h after treatment.

2.3. Cell processing

Bacteriological examination of all the lavages, by culture on blood agar plates (5% washed sheep erythrocytes) and aerobic incubation at 37 °C for 24 h, yielded invariably negative results. No bacteria were detected in any of the tested mammary lavages. Total mammary cell counts were determined using a haemocytometer. The Trypan Blue dye exclusion test demonstrated 95% cell viability. The cell suspensions were centrifuged at 4 °C and 200 × *g* for 10 min. One millilitre of supernatant was retained for resuspension of the pellet. The remaining supernatant was recanted.

2.4. Flow cytometry

An indirect staining technique was used. Mouse anti-bovine CD14 (VPM65, Serotec, Oxford, UK) diluted 1: 20 and fluorescein isothiocyanate-labelled swine anti-mouse immunoglobulin (SwM-FITC, Sevac, Prague, Czech Republic), diluted 1:360 were used as the primary and the secondary antibodies, respectively. Negative control samples were stained with the secondary antibody only.

The whole blood lysis technique was used for detection in peripheral blood samples. Fifty microlitres of blood were incubated in 12 × 75 mm tubes with the monoclonal antibody at room temperature for 15 min. Erythrocytes were lysed by adding 3 mL of a haemolytic solution (8.26 g of NH_4Cl, 1 g of $KHCO_3$, 0.037 g of Na_4 EDTA per 1 L of distilled water). The suspension was centrifuged, supernatant was removed, secondary antibody was added, and the tubes were incubated at 4 °C for 20 min. Thereafter, 3 mL of washing solution (1 g of $NaNO_3$ and 1.84 g of Na_4EDTA per 1 L of PBS) was added into the tubes. The suspensions were centrifuged, the super- natant was removed and the sediment was resuspended in the washing solution.

For the detection in mammary lavages, the cells were adjusted to 1×10^6/mL with the washing solution supplemented with 10% of heat-inactivated porcine serum. After 20 min, 50 μL of the cell suspension were incubated with the primary monoclonal antibody at 4 °C for 15 min. The cells were washed with the washing solution and centrifuged and the supernatant was removed. A secondary antibody was added and the tubes were incubated at 4 °C for 20 min. After another washing, the cells were resuspended in the washing solution.

The detection of CD14 was performed using the FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the CELLQuest™ software. At least 20 000 events were read. Gating of the individual cell populations was based on forward-scatter and side-scatter light characteristics and a acridin orange fluorescence as described by Hageltorn and Saad [5]. Briefly, the blood and mammary lavage samples were prepared by diluting 1000 μL of cell suspension with 10 μL of 0.0004% acridine orange and were analysed by flow cytometry. Data obtained were displayed in 2 dot plots (Fig. 1). The first dot plot was designed to provide two parameters differential leukocyte counts on the basis of cell size (FSC) and cytoplasmic granularity (SSC). On the first dot plot the clusters of individual cell types were marked and using the technique of backgating were displayed on the second dot plot. The second dot plot was designed to provide two parameters leukocyte differential count on the basis of

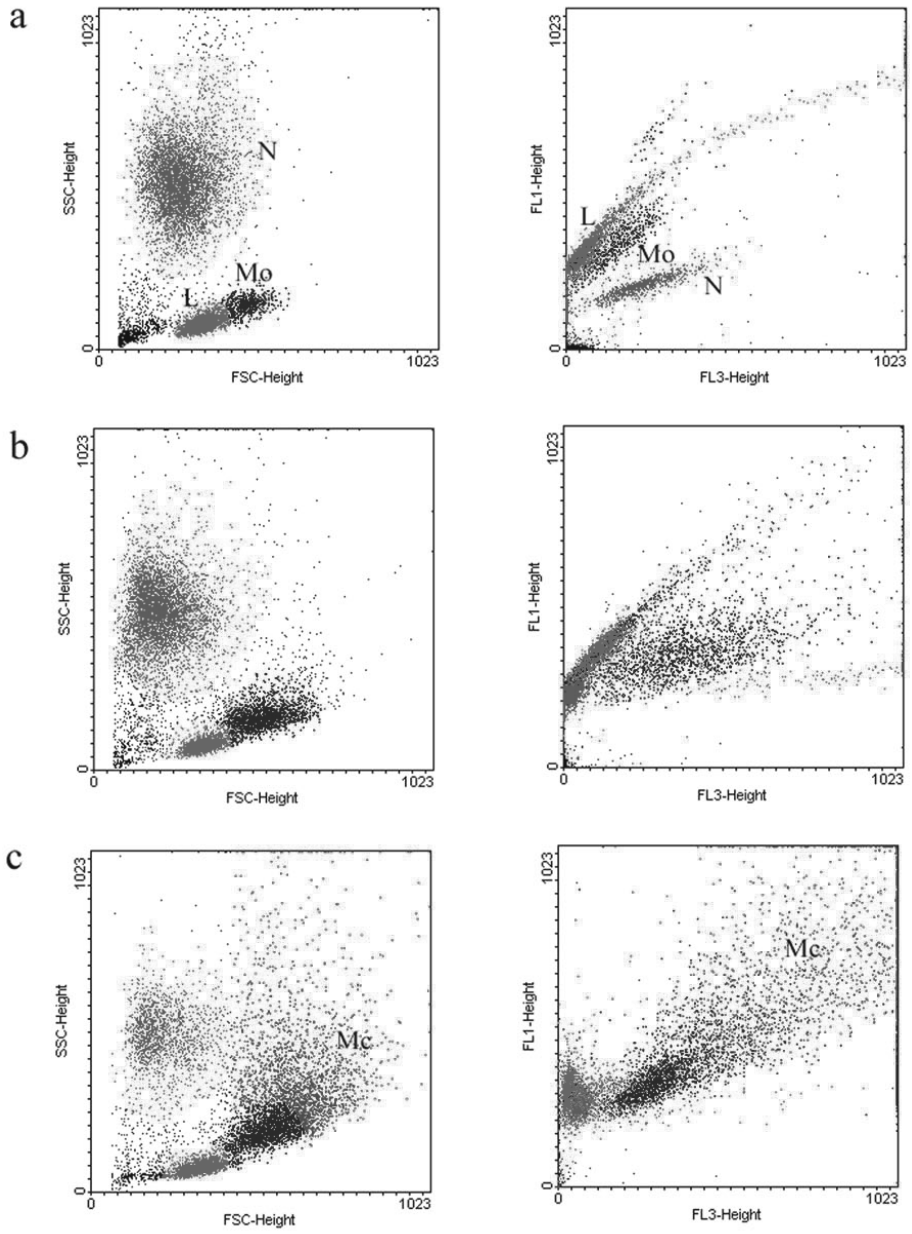

Figure 1. Dot plots of the blood and dot plots of the mammary lavages obtained from control and quarters 24 hours and 96 hours after induced mammary gland injury with LPS (1 μg/mL). Regions of individual cell types identified by light scatter parameters (left dot plots) and by acridine orange fluorescence (right dot plots) are shown. Neutrophils (N), lymphocytes (L), monocytes (Mo) and macrophages (Mc).

red (FL3) and green (FL1) acridine orange fluorescence. The clusters of individual cell types on the second dot plot were identified as described Hageltorn and Saad [5]. The forward and side scatter dot plot (the first dot plot) was used as a muster for later gating and analysis of CD14 on neutrophils, monocytes and macrophages. The percentage of CD14 positive cells was determined on a log-scale histogram. The cursor was set so that only 1% (in the peripheral blood) or 3% (in mammary lavages) of the events were to the right of it in the negative control. In the labelled samples, the percentage of events to the right of the cursor was considered positive.

2.5. Statistical analysis

The results underwent multifactorial analysis of variance for the determination of significant sources of variability. The significance of differences between timepoints during mammary gland injury caused by LPS and PBS was tested for means for total CD14+ neutrophil count, total CD14+ monocytes count and CD14+ macrophage count and differential CD14+ neutrophil, differential CD14+ monocyte count and differential CD14+ macrophage count was tested by the Scheffe method. Statistical analyses were carried out using STAT Plus software (Veterinary Research Institute, Brno, Czech Republic, 1992).

3. RESULTS

3.1. Differential leukocyte counts in the peripheral blood and intact mammary glands

The whole blood leukocyte population consisted of 38.5 ± 11.1% of neutrophils, 13.4 ± 3.5% of monocytes, and 48.1 ± 8.2% of lymphocytes. On the contrary, monocytes and macrophages predominated over other cells (mainly lymphocytes) and neutrophils in lavages from intact heifers mammary gland (Tab. I).

3.2. Responses of mammary gland leukocytes to PBS and LPS

Cell counts in mammary lavages before the induction of mammary gland injury were rather low. The intracisternal treatment with LPS or PBS resulted in significant changes in both total and differential cell counts. The total cell count increased significantly after 24 h and the response to LPS was significantly stronger than that to PBS ($P < 0.05$). At 48 h, the increase was followed by a decrease in total cell counts observed up to 96 h (Tab. I).

While macrophages predominated in the cell population before induction of mammary gland injury, the treatment with LPS or PBS resulted in a pronounced accumulation of neutrophils within 24 h (Tab. I). Both the total count and percentage of neutrophils decreased during the resolution phase (48 to 96 h) and this change was accompanied by increases in the percentages of monocytes, macrophages and lymphocytes. The clearance of neutrophils was more pronounced and more rapid after the treatment with PBS than after the treatment with LPS (Tab. I).

3.3. Evolution of CD14+ neutrophils, monocytes and macrophages during the induction and resolution of mammary gland injury

The percentages of CD14+ cells of circulating neutrophils and monocytes were 6.5 ± 3.2% and 57.3 ± 9.2%, respectively. CD14 cell surface receptors were expressed on neutrophils, monocytes and macrophages isolated from mammary glands too. Before the induction of mammary gland injury, the CD14+ cells were detected in 39.5 ± 6.6% of neutrophils, 56.4 ± 8.8% of monocytes,

Table I. Total and differential cell counts during mammary gland injury.

Time points (hours)	Number of cell ($\times 10^6$/mL)	Neutrophils (%)	Monocytes (%)	Macrophages (%)	Lymphocytes (%)
			PBS		
0	1.5 ± 0.3[a]	3.9 ± 1.4	13.9 ± 3.4	43.5 ± 4.5	38.7 ± 4.7
24	51.8 ± 23.5	86.3 ± 6.3	8.4 ± 2.6	1.9 ± 0.8	3.4 ± 2.9
48	12.1 ± 9.2	60.8 ± 8.8	12.8 ± 6.3	14.9 ± 8.1	11.5 ± 2.8
72	8.7 ± 5.9	15.5 ± 1.8	21.5 ± 7.8	36.6 ± 13.7	26.4 ± 3.8
96	3.1 ± 0.6	8.4 ± 1.5	22.8 ± 9.1	38.4 ± 13.3	30.4 ± 5.6
			LPS		
0	1.9 ± 0.6	4.1 ± 1.1	17.6 ± 4.9	45.1 ± 8.3	33.2 ± 6.5
24	148.8 ± 59.1	92.6 ± 3.7	4.2 ± 1.8	1.5 ± 0.7	1.7 ± 0.3
48	50.6 ± 29.4	78.3 ± 5.3	7.4 ± 2.9	5.9 ± 1.5	8.4 ± 2.8
72	31.4 ± 12.2	57.9 ± 11.8	16.1 ± 5.2	15.6 ± 6.8	10.4 ± 4.8
96	8.3 ± 3.9	11.8 ± 6.6	28.1 ± 9.3	39.7 ± 13.1	20.4 ± 7.1

[a] Mean ± SD (n = 5).

and 65.3 ± 9.5% of macrophages. The induction and resolution of mammary gland injury were accompanied by pronounced changes in number of CD14+ cells that were dependent on the inducing agent and time.

Neutrophils

The induction of mammary gland injury with PBS or LPS was followed by considerable changes in the total neutrophil count (Tab. I), total number of CD14+ neutrophils and percentage of CD14+ neutrophils (Fig. 2). An increase in total counts of CD14+ neutrophils was observed already at 24 h and was more marked after LPS than after PBS treatments ($P < 0.05$). This increase ceased at 48 h after PBS treatment, but continued thereafter to reach a 133 fold after the LPS treatment (Fig. 2). The number of CD14+ neutrophils decreased between 72 and 96 hours. The percentage of CD14+ neutrophils was almost the inverse of the total count of neutrophils during mammary gland injury. As shown in Figure 2, in the initial phase of mammary gland injury (24 h), CD14+ neutrophils comprised only 1.2% and 1.3% of the neutrophil population after treatment with PBS and LPS, respectively. The resolution was accompanied by an increase in the percentage of CD14+ neutrophils. The relative proportion of CD14+ neutrophils reached the pre-induction level after PBS treatment, but not after LPS treatment (Fig. 2).

Monocytes

As in neutrophils, the total count of CD14+ monocytes increased at 24 h and this increase was more marked after LPS than after PBS treatments ($P < 0.05$) (Fig. 3). A marked decrease without significant between-

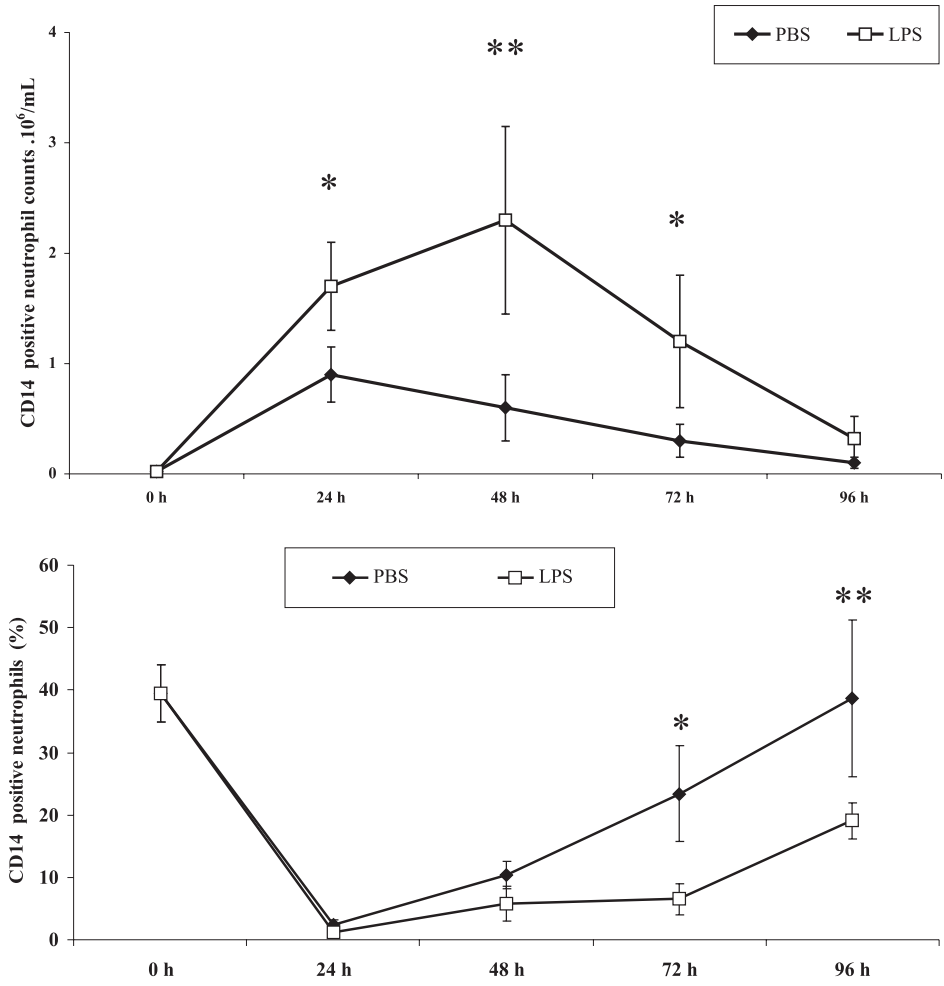

Figure 2. Numbers (a) and percentages (b) of CD14+ neutrophils (mean ± S.D.) in the total neutrophil population in mammary lavages collected at 24, 48, 72, and 96 h after intramammary instillation of PBS or LPS (1 μg/mL). Significant between-treatment differences are marked with asterisks (* $P < 0.05$; ** $P < 0.01$).

treatment difference was then observed between 48 and 96 h. Monocytes were the only cell types in which an increase in the percentage of CD14+ cells was observed in the initial phase of mammary gland injury (Fig. 3).

Macrophages

No significant change in the total count of CD14+ macrophages was observed at 24 h. An increase became apparent only at 48 h. This increase continued up to 72 h

after LPS, but not after PBS treatment (Fig. 4). The dynamics of the CD14+ macrophages percentage were similar to those described for CD14+ neutrophils (Fig. 2). A significant decrease in the percentage of CD14+ macrophages ($P < 0.01$) was observed 24 h after LPS treatment, but not after PBS treatment. The resolution of mammary gland injury (between 48 and 96 h) was characterised by a increase in proportion of CD14+ macrophages which was greater in the LPS-treated than PBS-treated mammary glands ($P < 0.01$).

4. DISCUSSION

The results of this study indicate that the resolution of mammary gland injury induced by PBS or LPS was accompanied by a marked activation of phagocytes, which was evident from an increase the proportion of CD14+ cells.

Intramammary treatment with LPS is known to induce an inflammatory response, the initial phase of which is characterised by a massive influx of leukocytes [14, 16, 17, 26] and production of pro-inflammatory cytokines (interleukins 1 and 6, TNF-α) and a C5a fragment of complement [17, 21, 22]. Influx of leukocytes can also be induced by intramammary treatment with PBS [4, 20]. The influx in which neutrophil leukocytes predominate, culminates within 24 h. Mammary gland injury is resolved after 72 to 96 h and the major role is played by apoptosis and phagocytosis of apoptotic neutrophils by mononuclear phagocytes [23, 24]. The elimination of neutrophils is associated with shifts in differential counts of other cell types. The percentages of monocytes, macrophages and lymphocytes increase and differential cell counts tend to return to pre-induction values. The resolution of mammary gland injury was more rapid after PBS treatment than after LPS treatment. This result is consistent with our earlier findings [23, 24].

Our results further indicate that, before mammary gland injury induction, the expression of CD14 was significantly lower on blood neutrophils than on mammary gland neutrophils. This finding is consistent with the data published by Paape et al. [14] on neutrophils of the lactating bovine mammary gland and is probably due to the microenvironment of the cavity system of the mammary gland, because neutrophils dispose of an intracytoplasmatic CD14 pool in secretion vesicles [3]. The microenvironmental factors are responsible for the time-dependent translocation of CD14 from the intracytoplasmatic pool to the cell surface [14].

The initial phase of mammary gland injury was characterised by distinctive shifts in percentages and total counts of CD14+ neutrophils. The marked decrease in the percentage may have been due to a release of CD14 molecules from the cell surface [14]. The purpose of this down-regulation in LPS-induced mammary gland injury is to obviate excessive production of interleukin-1 and TNF-α[26], because the released soluble CD14 neutralises the LPS-LBP complex and thus blocks continuing instigation of the inflammatory response [10].

The decrease in the percentage of CD14+ neutrophils was accompanied by an increase in total counts of neutrophils which reached peak values of 1000 fold and 2900 fold in PBS-induced and LPS-induced mammary gland injury, respectively. This increase may have partly resulted from the migration of circulating CD14+ neutrophils, because this population included 6.5% of CD14+ cells. Our findings are fully consistent with the data published by Paape et al. [14].

Both CD14+ and total neutrophil counts decreased markedly during the resolution of mammary gland injury. This decrease became apparent at 48 h in PBS-induced and at 72 h in LPS-induced mammary gland injury. These changes closely correlate with the clearance of apoptotic neutrophils, as

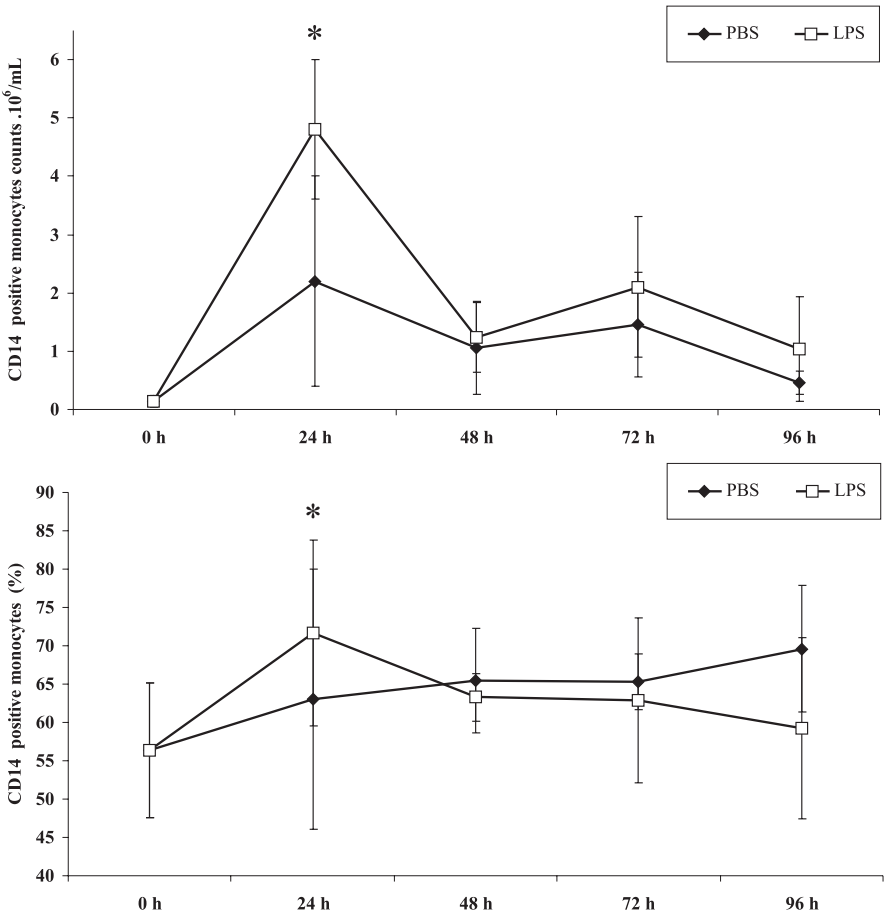

Figure 3. Numbers (a) and percentages (b) of CD14+ monocytes (mean ± S.D.) in the total monocytes population in mammary lavages collected at 24, 48, 72, and 96 h after intramammary instillation of PBS or LPS (1 μg/mL). Significant between-treatment differences are marked with asterisks (*$P < 0.05$).

determined in our previous paper in which the highest counts of apoptotic neutrophils and myeloperoxidase-positive mononuclear phagocytes were found at 48 and 72 h in PBS-induced and LPS-induced mammary gland injury, respectively [23, 24]. This difference can be attributed to LPS which prolonged the influx of neutrophils and delayed the onset of neutrophil apoptosis. Interestingly enough, the expression of CD14 was distinctly higher in apoptotic than in intact neutrophils (unpublished data). This finding is consistent with the data of Mills et al. [12] who stated that an increase in CD14+ HL60 cells was accompanied by a decrease in viability and an

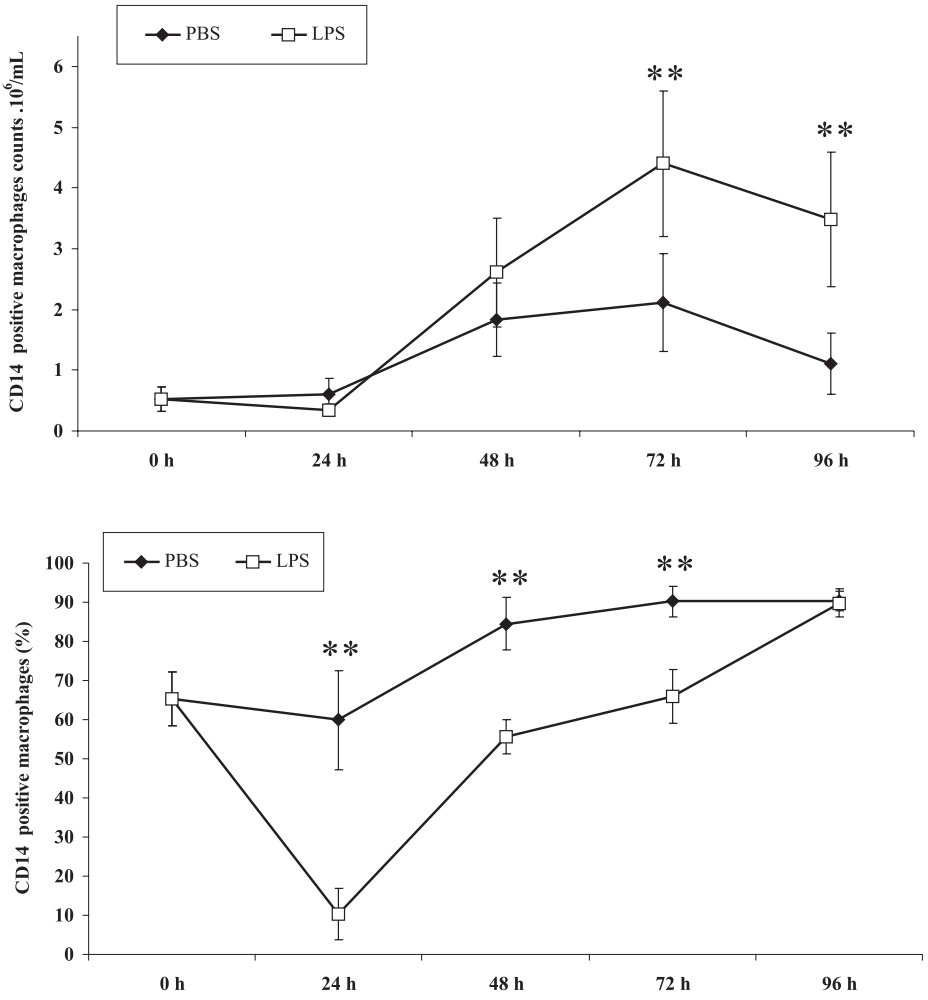

Figure 4. Numbers (a) and percentages (b) of CD14+ macrophages (mean ± S.D.) in the total macrophages population in mammary lavages collected at 24, 48, 72, and 96 h after intramammary instillation of PBS or LPS (1 μg/mL). Significant between-treatment differences are marked with asterisks (*$P < 0.05$; **$P < 0.01$).

increase in positivity for Annexin V. Considering this finding and our data on the expression of CD14 on neutrophils, we can conclude that clearance of apoptotic neutrophils coincides with a decrease in CD14+ neutrophil counts. On the contrary, the percentage of CD14+ neutrophils increased during the resolution of mammary gland injury, tending to return to pre-induction values.

CD14 receptor is a marker for monocytes and macrophages, although all monocytes and macrophages do not express CD14. Blood monocytes express either high and low CD14 and population of inflammatory monocytes express low CD14 [15].

Also macrophages express CD14 low (alveolar macrophages) and high (pleural and peritoneal macrophages) [6]. In this paper we observed that not all monocytes and macrophages express CD14 receptor during initiation and resolution of mammary gland injury. This can be caused by contamination of monocytes region by large lymphocytes. However, in our preliminary study (unpublished data), we analysed contamination of monocytes cluster by large lymphocytes in cell population from blood and mammary gland (5 heifers – 20 mammary glands). Lymphocytes were identified by signs typical for this cell types: CD2, CD3, pan B. Contamination was < 10% in blood and < 5% in mammary gland. We exclude significant contamination of the monocyte and macrophage population by large lymphocytes, because in timepoints with the highest proportion of lymphocytes (72 and 96 h after treatment with PBS and LPS) was not detected significant decrease of relative proportion of CD14+ monocytes and CD14+ macrophages.

Monocytes migrate into the cavity system of the mammary gland in the initial phase of LPS-induced mammary gland injury and are activated by interaction with LPS. This is evident from differences in total counts and percentages of CD14+ monocytes at 24 h after the induction of mammary gland injury. The up-regulating effect of LPS on mononuclear phagocytes was described by Landmann et al. [9] and Chen et al. [2]. In vitro experiments on human mononuclear phagocytes demonstrated that the expression of CD14 increased at low (0.1 to 1.0 ng/mL) and variably decreased at high (100 ng/mL and higher) LPS concentrations. In our experiments, the induction of mammary gland injury with LPS at 1 μg/mL resulted in the increased of proportion of CD14+ monocytes at 24 h. On the monocytes of the bovine mammary gland, the affinity of CD14 towards LPS may be lower than that of human CD14, as indicated by the results of studies in bovine alveolar mononuclear phagocytes published by Jungi et al. [8]. Our results may also have been influenced by the dilution effect of exudation accompanying the influx. Generally, such discrepancies are attributable to differences between in vivo and in vitro experimental conditions.

As stated above, monocytes act as effective scavengers during the resolution of experimentally induced mammary gland injury. Intensive phagocytosis of apoptotic neutrophils at 48 h after mammary gland injury induction, confirmed by staining for myeloperoxidase, was demonstrated in our previous studies [23, 24]. Between 48 and 72 h, the percentage of myeloperoxidase-positive monocytes in the cavity system of the mammary gland exceeded 40 and morphologically distinct, recently phagocytised neutrophils were observed in 1/4 of them. As a consequence of phagocytosis, monocytes increase in size, acquire rounded shapes and in the FACS pattern shift towards the region of the macrophages cluster. Hence, the loss of CD14+ mono- cytes and the coincident increase in CD14+ macrophages at 48 h after influx induction are not surprising.

The initiation and resolution of LPS-induced mammary gland injury was characterised by a rapid decrease in the proportion of CD14+ macrophages that was indicative of a loss of macrophages activation. Surprisingly, the proportion of CD14+ monocytes increased and the proportion of CD14+ macrophages decreased at 24 h after treatment with LPS. Causes of this difference in responses to LPS are unclear. One of them may be a change in the ability of the interaction with LPS during the transformation of monocytes into macrophages [10]. Similar results were reported for murine recruited alveolar monocytes and resident macrophages [11].

The results of our study indicate activation of phagocytes during resolution of LPS-induced mammary gland injury

manifested by increasing proportion of CD14+ cells. The increased proportion of CD14+ neutrophils was apparently due rather to the action of intrinsic factors than to the effect of LPS, because migration, induced by LPS or PBS, did not increase CD14 expression on neutrophils. On the contrary, monocytes are activated by LPS as was evident from an increased proportion of CD14+ cells after LPS-induced migration. In consequence of the scavenging activity during the resolution of mammary gland injury, monocytes acquire morphological characteristics of macrophages that also show activation at the onset of resolution. The activation of mononuclear phagocytes during the resolution of LPS-induced mammary gland injury is indicative of intensive reparation processes in the damaged mammary gland consisting above all in the removal of neutrophils. Generally, this process can be regarded as an effective mechanism preventing the development of mammary gland injury into chronic inflammation.

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