# CD14 expression, apoptosis and necrosis in resident and inflammatory macrophages from virgin bovine mammary gland

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**ABSTRACT**: This paper investigates the association between expression of CD14 and occurrence of apoptosis in blood monocytes, resident ($_{RES}$MAC) and inflammatory macrophages from heifer mammary glands after infusion of PBS ($_{INF}$MAC$_{PBS}$) or LPS ($_{INF}$MAC$_{LPS}$). Resident macrophages ($_{RES}$MAC) were obtained before, and inflammatory macrophages ($_{INF}$MAC$_{PBS}$ and $_{INF}$MAC$_{LPS}$) 24 h after, induction of an inflammatory response using phosphate buffered saline (PBS) and lipopolysaccharide (LPS) in mammary glands of unbred heifers. Cell samples were analysed for differential counts, CD14 expression, apoptosis and necrosis using flow cytometry. *In vitro* cultivation led to a decrease in the proportion of living cells and to an increase in the proportion of apoptotic and necrotic cells in all macrophages and blood monocytes. In CD14$^+$ macrophages, the proportions of live cells increased and proportions of apoptotic and necrotic cells decreased after *in vitro* cultivation. We observed in CD14$^-$ macrophages and monocytes that the proportions of live cells decreased and proportions of apoptotic and necrotic cells increased after *in vitro* cultivation. Our experiments confirm that the expression of CD14 in bovine mammary gland macrophages and blood monocytes is associated with cell viability.

**Keywords**: virgin bovine mammary gland; *in vitro;* macrophages; CD14; apoptosis; cytokines; lipopolysaccharide

**List of abrevations**

**ANOVA** = analysis of variance, **FACS** = flow cytometry**, FCM** = flow cytometry**, FITC** = fluorescein isothiocyanate, **FL** = channel, **IL** = interleukine, $_{INF}$**MAC**$_{LPS}$ = inflammatory macrophages, $_{INF}$**MAC**$_{PBS}$ = inflammatory macrophages, **LBP** = lipopolysaccharide-binding protein, **LPS** = lipopolysaccharide, **PAMPs** = pathogen associated molecular patterns, **PI** = propidium iodide, **PMN** = polymorphonuclear leukocyte, $_{RES}$**MAC** = resident macrophages, **rPE** = r-phycoerythrin, **TGF-β** = transforming growth factor, **TLR** = Toll-like receptors, **TNF-α** = tumour necrosis factor alpha

Macrophages are the predominant cell type in healthy mammary gland of heifers (Wardley et al. 1976). Macrophages have phagocytic functions and additional regulatory roles, including regulation of homeostasis, cellular communication, and induction and termination of local immune responses.

The first step in an immune response is the recognition of invading pathogens through receptors, which are expressed on the surface of macrophages. Toll-like receptors (TLR) are one family of receptors that interact with a broad family of common pathogen associated molecular patterns (PAMPs) from different microbial invaders. The PAMPs (lipopolysaccharide, peptidoglycan and lipoteichoic acid) are capable of initiating the immune activation of monocyte-derived cells (Mogensen 2009). Recognition of these PAMPs is facilitated by accessory molecules: lipopolysaccharide-binding protein (LBP) and the CD14 receptor.

CD14, a glycosylphosphatidylinositol-linked receptor, is present on mononuclear cells (mono-

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cytes, macrophages) and, to a lesser degree, on polymorphonuclear leukocytes (PMN) (Landmann et al. 1991). Binding of lipopolysaccharide (LPS) to CD14 via LBP initiates signal transduction through TLR-4 and results in release of pro-inflammatory cytokines by macrophages (TNF-α, IL-1β, IL-6 and IL-8). These cytokines trigger PMN migration from the blood to the site of injury, inducing an innate immune response against invading bacteria (Murray and Wynn 2011). Therefore, CD14 serves as a key receptor and recently it has been the subject of intensive research in the bovine mammary gland. Differential expression of CD14 has been observed in freshly migrated inflammatory macrophages (Paape et al. 1996; Sladek et al. 2002), in resident macrophages from alveoli (Leitner et al. 2003; Sladek and Rysanek 2006) and from surrounding tissue (Leitner et al. 2003; Dallard et al. 2009), and finally in blood monocytes (Paape et al. 1996).

In blood monocytes, the expression of CD14 is strongly associated with cell viability. In fact, enzymatic removal of membrane-bound CD14 by phosphatidylinositol-specific phospholipase C has been shown to evoke programmed cell death, or apoptosis. On the other hand, activating stimuli (IL-1, TNF-α, LPS), which lead to enhanced CD14 expression, rescue blood monocytes from apoptosis (Heidenreich et al. 1997).

The recruited macrophages from blood monocytes also undergo apoptosis and are removed by resident phagocytes, thus further helping in the resolution of inflammation (Kolaczkowska et al. 2010; Sladek and Rysanek 2010; Janssen et al. 2011; Fischer et al. 2013; Periasamy et al. 2013). However, the relationship between apoptosis and CD14 expression is not completely understood in terminally differentiated macrophages after their migration into inflamed tissues. Therefore, the question remains, whether we can expect anti-apoptotic effects of CD14 in these macrophages *in situ;* it also remains to be elucidated whether the longer life expectancy of macrophages *in situ* is determined by resistance to stimuli that elicit apoptosis of blood monocytes *in vitro* (Kiener et al. 1997). This is very important because *in situ* macrophages produce both pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, IL-12) and anti-inflammatory cytokines (IL-10, TGF-β) in response to microbial stimuli during intramammary infection of dairy cows (Bannerman 2009). Specifically, the concentrations of IL-1 and TNF-α, which significantly affect CD14 expression and thus also apoptosis, are increased within 18 h of experimental intramammary *E. coli* infection (Shuster et al. 1995; Bannerman et al. 2004).

Therefore, the aim of the present study was to evaluate how the expression of CD14 is associated with cell viability in recruited and resident macrophages obtained before and after an inflammatory response induced by CD14-dependent (LPS) and -independent agents (PBS). Because the relationship between apoptosis and CD14 expression is known in blood monocytes, in addition to mammary gland macrophages we also analysed blood monocytes in this study and used this cell population as a baseline for comparison.

## MATERIAL AND METHODS

**Animals**. The experiments were conducted on ten virgin, clinically healthy, Holstein × Bohemian Red Pied crossbred heifers, aged 15 to 18 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 protocol. All experimental procedures were approved by the Central Commission for Animal Welfare of the Czech Republic. All heifers were free of intramammary infections, as demonstrated through bacteriological examination of mammary lavages.

**Blood sampling, isolation and processing of blood leukocytes**. Blood (10 ml) was drawn by venipuncture from the jugular vein of the experimental heifers into a sterile flask containing 1000 IU heparin (Leciva a.s., Dolni Mecholupy, Czech Republic) in 10 ml of PBS (Sigma, Saint Louis, MO, USA).

Isolation of monocytes from whole blood samples was carried out using FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA, USA) and a procedure previously described (Hodge et al. 1999). Briefly, each blood sample (500 µl) was labelled (for details, see text below) and kept in darkness at room temperature for 15 min. After adding 2 ml of FACS Lysing Solution, the sample was resuspended and centrifuged for 5 min at 500 × g. Then, the supernatant was decanted and 2 ml of PBS were added. Another 5-min centrifugation at 500 × g followed, the supernatant was then removed, and the sediment was resus-

pended in 500 µl of RPMI medium (RPMI 1640 medium, Sigma, Aldrich, USA, supplemented with 100 000 IU/l penicillin, 100 mg/l streptomycin and 4 mg/l gentamicin with 10% foetal calf serum from Invitrogen, Carlsbad, USA).

**Collection of macrophages from mammary gland**. Three samples of mammary gland macrophages were obtained in this study. The first sample was from the untreated mammary gland of heifers (resident macrophages [$_{RES}$MAC]), the second from mammary gland following PBS infusion (inflammatory macrophages [$_{INF}$MAC$_{PBS}$]), and the third following LPS infusion (inflammatory macrophages [$_{INF}$MAC$_{LPS}$]).

A modified urethral catheter (AC5306CH06, Porges S.A., France) was inserted into the teat canal of each quarter after thorough disinfection of the teat orifice with 70% ethanol. Each mammary quarter was infused with 20 ml PBS (dissolved in apyrogenic distilled water, Sigma, St. Louis, MO, USA), massaged, and then the PBS containing the resident cell population was retrieved through the catheter. The lavage volumes were 15–20 ml.

Immediately after harvesting resident cells, the mammary glands of five heifers were infused as before with 20 ml PBS. The mammary glands of the remaining five heifers were infused with 10 mg of LPS (LPS of *Escherichia coli* serotype O128:B12, Sigma, St. Louis, MO, USA) diluted in 20 ml PBS. Samples of the mammary gland inflammatory cell populations were obtained by lavage 24 h after treatment. The lavage volumes were 15–20 ml.

**Isolation and processing of resident and inflammatory macrophages**. Immediately after harvesting, all obtained lavages were bacteriologically examined by culture on blood agar plates (5% washed sheep erythrocytes) and aerobic incubation at 37 °C for 24 h. The results in all cases were negative.

Total mammary cell counts in lavages were determined using a Fossomatic 90 apparatus (Foss Electric, Denmark) and the procedure recommended by the International Organization for Standardization (2006).

Next, both samples of resident and inflammatory cells (15–20ml) from lavages were centrifuged at 4 °C and 200 × *g* for 10 min. One millilitre of the supernatant was retained for resuspension of the pellet. The remaining supernatant was decanted. All of the samples were adjusted (5 × $10^6$ cells/ml) in RPMI 1640.

***In vitro* cultivation of blood monocytes and mammary gland macrophages**. The processed blood monocytes and mammary gland macrophages were divided into two parts. The first part was immediately analysed as a fresh population. The remaining samples were incubated *in vitro*. The adjusted blood and mammary gland cell suspensions were inserted into Corning microplates (Costar Ultra Plates, Myriad Industries, San Diego, CA, USA) and were incubated for three and six hours at 37 °C in an incubator with a 5% $CO_2$ atmosphere in accordance with Newman et al. (1982). The fresh and *in vitro* cultivated cell populations were analysed by flow cytometry.

**Flow cytometry analysis**. Flow cytometry (FCM) analysis was used for determining the differential counts, detecting apoptosis and necrosis in cells, and detecting CD14 expression. The instrument for FCM was set to analyse 20 000 cells per sample. Final dot plots were evaluated qualitatively and quantitatively using CellQuest software (Becton Dickinson, Mountain View, CA, USA).

*Differential cell count.* Percentages of individual cell types – granulocytes, monocytes, macrophages and lymphocytes – were read from forward-scatter versus side-scatter dot plots as described previously (Sladek et al. 2002) (Figure 1).

*Detection of apoptosis and necrosis.* Apoptotic and necrotic monocytes and macrophages were analysed after simultaneous staining with Annexin-V labelled with FITC and propidium iodide (PI) as described by Vermes et al. (1995). The commercial AnnexinV-FLUOS Staining Kit (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, 500 ml of the incubation buffer (10mM HEPES/NaOH, pH 7.4; 140mM NaCl; 2.5mM $CaCl_2$) was mixed with 10 ml of PI and 10 ml of FITC-Annexin-V solutions to prepare the working solution. The cell suspension was adjusted to 1 x $10^6$/ml in 100 ml of fresh incubation buffer containing PI and FITC-Annexin-V. The suspension was analysed using FCM after 15 min of incubation at room temperature.

*Detection of CD14 expression.* We used tricolour labelling to detect CD14 expression in healthy, apoptotic and necrotic monocytes and macrophages. The cells were washed in solution supplemented with 10% heatinactivated porcine serum. After 20 min, 50 ml of the cell suspension was incubated with the primary monoclonal antibody (mouse anti-ovine CD14, VPM65, Serotec, Oxford, UK) diluted 1 : 20 at 4 °C for 15 min. The cells were washed with the washing solution and centrifuged after

which the supernatant was removed. A secondary antibody (r-phycoerythrin, rPE labelled swine anti-mouse IgG1, SouthernBiotech, Birmingham, Alabama, USA) diluted 1 : 360 was added and the tubes were incubated at 4 °C for 20 min. After another wash step, the cells were resuspended in the washing solution. Negative control samples were stained with the secondary antibody only. The labelled samples and controls were immediately stained with FITC (FL1) and PI (FL3) to demonstrate the association between cell death and CD14 expression (FL2 – rPE) and evaluated by FCM.

*Method for compensation of AnnexinV-FITC, rPE and PI in flow cytometry.* The FL2-FL3 channel and FL3-FL2 channel are critical for three colour setups using rPE and PI. Since PI from FL3 channel bleeds into FL2 channel it must be compensates. Therefore, we used control samples with PI and no rPE and samples with rPE but no PI to show that clean FL2 and FL3 signals were obtained in these control samples (Figure 2). The strategy for FCM analysis after compensation was to identify:
CD14 positive cells → live ($CD14^+/AnnexinV^-/PI^-$), apoptotic ($CD14^+/AnnexinV^+/PI^-$), and necrotic cells ($CD14^+/AnnexinV^+/PI^+$).
CD14 negative cells → live ($CD14^-/AnnexinV^-/PI^-$), apoptotic ($CD14^-/AnnexinV^+/PI^-$), and necrotic cells ($CD14^-/AnnexinV^+/PI^+$).

Debris was always excluded from the enumeration due to the possible CD14 positivity of epithelial cells (Strandberg et al. 2005).

**Statistical analysis**. One way analysis of variance (ANOVA, Scheffe's test) was used to evaluate the significance of differences in the proportions of $_{RES}MAC$, $_{INF}MAC_{PBS}$, $_{INF}MAC_{LPS}$, $CD14^+$, apoptotic and necrotic monocytes and macrophages. The statistical analysis was used to determine significant time-point and between treatment differences in all *in vitro*-cultivated cell populations. For this purpose, all percentage values were calculated from the same total values of cells in each sample (i.e. $2 \times 10^4$ cells). Statistical analyses were carried out using GraphPad Prism ver. 5.01 (GraphPad Software, La Jolla, CA, USA).

## RESULTS

### Mammary gland macrophages *in situ*

In untreated mammary glands, we observed $_{RES}MAC$ to be the dominant cell population, comprising nearly two-thirds of all cells and followed by lymphocytes and neutrophils. Based on forward-scattered and side-scattered light parameters on dot plots in FCM and morphology under light microscopy, it was possible to distinguish two forms of $_{RES}MAC$: monocyte-like macrophages and vacuolised macrophages (Figure 1A). Vacuolised macrophages were slightly dominant (29.7 ± 5.5%) in untreated mammary glands in comparison to monocyte-like macrophages (27.3 ± 7.6%).

Intramammary application of PBS or LPS resulted in an inflammatory response characterised by an influx of inflammatory cells, of which neutrophils were the dominate cell type (Figure 1C). The cell populations contained approximately 20% $_{INF}MAC_{PBS}$ and 6% $_{INF}MAC_{LPS}$, respectively. In contrast to untreated mammary glands, monocyte-like macrophages were the dominant form in PBS-treated mammary glands (15.5 ± 4.2%) and in LPS-treated mammary glands (3.1 ± 2.1%).

Table 1. The proportions of $CD14^+$, apoptotic and necrotic cells in fresh populations of resident and inflammatory macrophages and blood monocytes and after cultivation *in vitro*

| | | $CD14^+$ cells | | Apoptotic cells | | Necrotic cells | |
|---|---|---|---|---|---|---|---|
| | | 0 h | 6 h | 0 h | 6 h | 0 h | 6 h |
| $_{RES}MAC$ | monocyte-like cells | 53.1 ± 10.4 | 64.4 ± 7.5 | 6.6 ± 1.6 | 14.4 ± 3.6** | 2.1 ± 0.4 | 8.9 ± 1.9** |
| | vacuolised cells | 78.5 ± 10.5 | 89.9 ± 9.7 | 22.9 ± 4.7 | 27.2 ± 4.4 | 16.5 ± 3.3 | 30.1 ± 3.9** |
| $_{INF}MAC_{PBS}$ | monocyte-like cells | 60.7 ± 12.5 | 70.1 ± 14.5 | 3.9 ± 2.1 | 12.5 ± 4.2** | 3.8 ± 1.2 | 10.2 ± 4.8** |
| | vacuolised cells | 73.1 ± 6.3 | 74.1 ± 7.8 | 9.1 ± 2.6 | 17.9 ± 3.1* | 11.1 ± 2.9 | 30.6 ± 4.9** |
| $_{INF}MAC_{LPS}$ | monocyte-like cells | 39.1 ± 9.8 | 57.3 ±9.2* | 4.9 ± 2.3 | 8.4 ± 1.6* | 10.3 ± 4.1 | 23.4 ± 5.2** |
| | vacuolised cells | 68.1 ± 4.3 | 79.2 ± 6.2** | 6.1 ± 2.8 | 9.4 ± 4.5 | 15.3 ± 3.8 | 40.9 ± 6.3** |
| Blood monocytes | | 52.3 ± 4.2 | 26.1 ± 7.4** | 1.2 ± 0.4 | 21.5 ± 6.8** | 1.9 ± 1.3 | 8.7 ± 2.2** |

Data are means ± SD in percentages and significant differences are marked by asterisks (*$P < 0.05$, **$P < 0.01$; Scheffe's method). The comparisons were made among 0 h samples and 6 h samples after cultivation *in vitro*

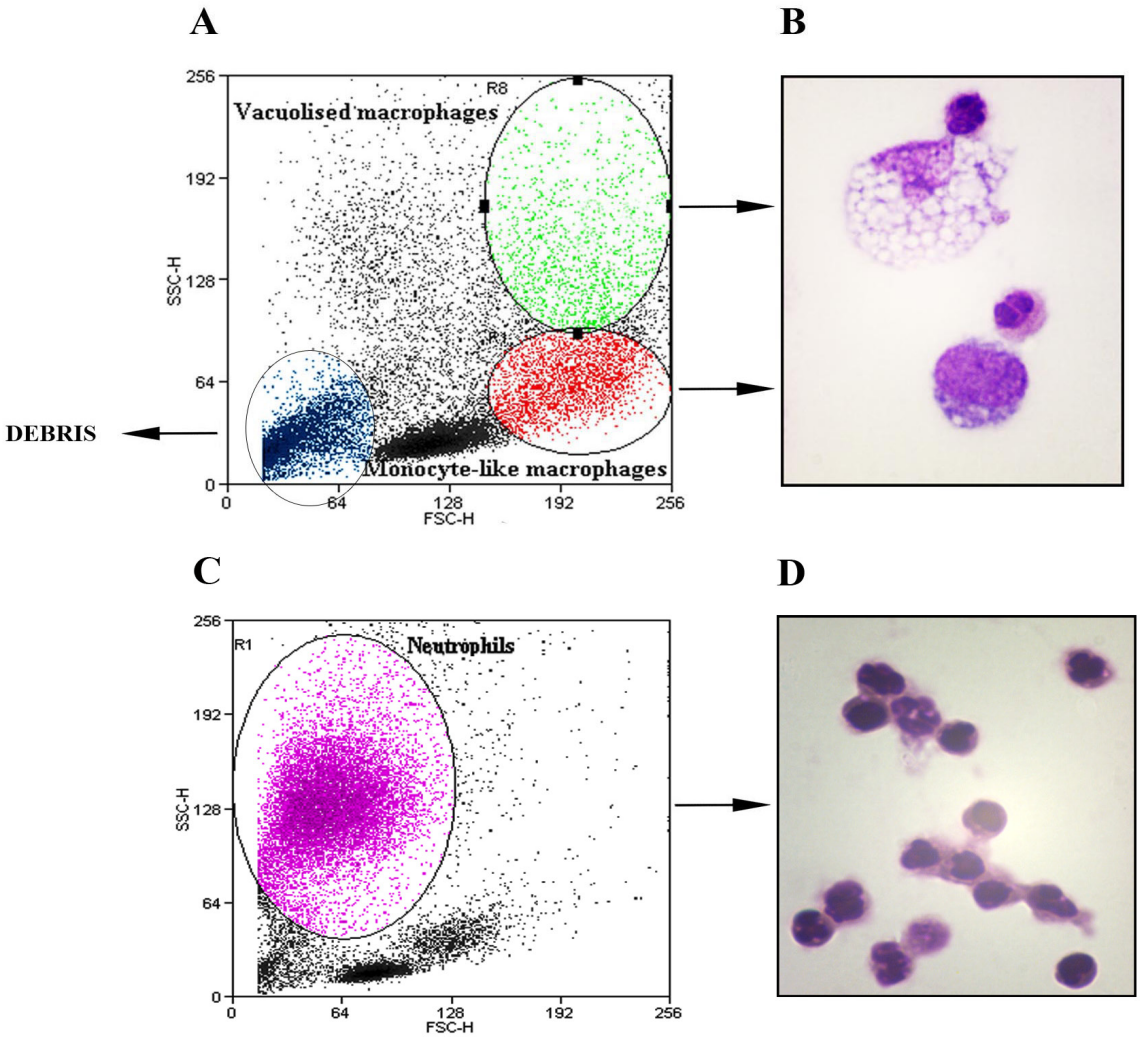


Figure 1. Gating strategy for monocyte-like and vacuolised resident macrophages and neutrophils on representative dot plots. The higher dot plot shows the distribution of cells differentiated by their forward-scatter (FSC) and side-scatter (SSC) parameters, with the monocyte-like $_{RES}$MAC (red) and vacuolized $_{RES}$MAC (green) populations highlighted (**A**). Cells obtained from untreated mammary glands. The lower dot plot shows the distribution of cells differentiated by their FSC and SSC parameters, with the neutrophils (magenta) population highlighted (**C**). Cells obtained 24 h after intramammary application of LPS. Monocyte-like $_{RES}$MAC and vacuolised $_{RES}$MAC are evident together with two neutrophils (**B**) and a few neutrophils (**D**) are also seen under the light microscope. May-Grünwald Giemsa stain (Pappenheim method). Magnification 1000.

The fresh populations of $_{RES}$MAC, $_{INF}$MAC$_{PBS}$ and $_{INF}$MAC$_{LPS}$ contained different proportions of CD14$^+$, apoptotic and necrotic cells (Table 1). Higher proportions of CD14$^+$, apoptotic and necrotic cells were observed in vacuolised $_{RES}$MAC. Conversely, a lower proportion of CD14$^+$ cells was observed in monocyte-like $_{INF}$MAC$_{LPS}$, apoptotic cells in monocyte-like $_{INF}$MAC$_{PBS}$, and finally necrotic cells in monocyte-like $_{RES}$MAC (Table 1).

In freshly isolated blood monocytes, more than half the cells were CD14$^+$. Apoptosis and necrosis were observed at very low levels (Table 1).

## Cultivation of mammary gland macrophages and blood monocytes *in vitro*

*In vitro* cultivation altered the proportions of CD14$^+$, apoptotic and necrotic cells in all populations of macrophages and blood monocytes (Table 1).

**CD14 expression**. The proportions of CD14$^+$ cells increased during cultivation in all macrophage populations. However, when we compared time points 0 h and 6 h, significant differences were observed only in monocyte-like $_{INF}$MAC$_{LPS}$ ($P < 0.05$) and vacuolised $_{INF}$MAC$_{LPS}$ ($P < 0.01$) (Table 1).

Table 2. *In vitro* time-dependent changes in proportions of live, apoptotic and necrotic cells in populations of CD14 positive macrophages and CD14 positive blood monocytes after 6 h cultivation measured by tricolour immunofluorescence using flow cytometry

| $CD14^+$ cells | | $CD14^+$/live cells | | $CD14^+$/apoptotic cells | | $CD14^+$/necrotic cells | |
|---|---|---|---|---|---|---|---|
| | | 0 h | 6 h | 0 h | 6 h | 0 h | 6 h |
| $_{RES}MAC$ | monocyte-like cells | 86.1 ± 3.9 | 93.4 ± 2.8* | 10.4 ± 4.5 | 3.4 ± 1.2** | 3.5 ± 1.1 | 3.2 ± 1.2 |
| | vacuolised cells | 58.4 ± 7.3 | 79.3 ± 5.5** | 31.7 ± 4.1 | 14.6 ± 4.5** | 9.9 ± 3.3 | 6.1 ± 3.9 |
| $_{INF}MAC_{PBS}$ | monocyte-like cells | 75.3 ± 8.2 | 88.8 ± 5.8** | 14.8 ± 5.3 | 7.4 ± 4.2* | 9.9 ± 3.2 | 3,8 ± 1.8 |
| | vacuolised cells | 65.3 ± 8.2 | 81.8 ± 4.1* | 23.1 ± 4.8 | 11.4 ± 3.9** | 11.6 ± 5.1 | 6.8 ± 2.9 |
| $_{INF}MAC_{LPS}$ | monocyte-like cells | 73.4 ± 7.1 | 82.8 ± 6.3* | 16.2 ± 3.3 | 9.8 ± 3.7** | 10.4 ± 4.4 | 7.4 ± 3.2 |
| | vacuolised cells | 36.8 ± 4.3 | 49.2 ± 13.7* | 32.3 ± 12.2 | 28.1 ± 9.5 | 30.9 ± 4.8 | 22.7 ± 7.2 |
| Blood monocytes | | 89.9 ± 4.3 | 60.2 ± 8.6** | 7.2 ± 1.7 | 26.6 ± 6.8** | 2.9 ± 0.7 | 13.2 ± 3.9** |

Data are means in percentages, measured at 0 h and 6 h, and significant differences are marked by asterisks (*$P < 0.05$, **$P < 0.01$; Scheffe's method). The comparisons were made among 0 h samples relative to 6 h samples after cultivation *in vitro*

Significant between-treatment differences were observed between vacuolised $_{RES}MAC$ and monocyte-like $_{INF}MAC_{PBS}$ ($P < 0.01$) and $_{INF}MAC_{LPS}$ ($P < 0.01$).

**Apoptosis**. A similar phenomenon was observed for apoptosis, which increased in all macrophage populations. The only instance in which the between-time point differences observed were not significant was in vacuolised $_{RES}MAC$ and $_{INF}MAC_{LPS}$ (Table 1). Significant between-treatment differences were observed between vacuolised $_{RES}MAC$ and remaining macrophage populations ($P < 0.01$) after 6 h of cultivation.

**Necrosis**. When we compared the proportions of necrotic cells at 0 h and 6 h time points, significant differences ($P < 0.01$) were observed in all populations of macrophages and blood monocytes (Table 1). Significant between-treatment differences ($P < 0.01$) were observed in vacuolised $_{INF}MAC_{LPS}$ in comparison to the other groups of macrophages and monocytes.

In blood monocytes, by contrast, a significant decrease in $CD14^+$ cells ($P < 0.01$) was observed along with a significant increase in apoptotic and necrotic cells ($P < 0.01$) after 6 h of cultivation (Table 1).

## Tricolour labelling of macrophages and blood monocytes

Tricolour labelling analysis revealed differing proportions of living, apoptotic and necrotic cells in the populations of $CD14^+$ macrophages and

Table 3. *In vitro* time-dependent changes in proportions of live, apoptotic and necrotic cells in populations of CD14 negative macrophages and CD14 negative blood monocytes after 6 h cultivation measured by tricolour immunofluorescence using flow cytometry

| $CD14^-$ cells | | $CD14^-$/live cells | | $CD14^-$/apoptotic cells | | $CD14^-$/necrotic cells | |
|---|---|---|---|---|---|---|---|
| | | 0 h | 6 h | 0 h | 6 h | 0 h | 6 h |
| $_{RES}MAC$ | monocyte-like cells | 93.4 ± 4.5 | 90.4 ± 6.5 | 2.7 ± 1.5 | 4.3 ± 2.8 | 3.9 ± 2.7 | 5.3 ± 2.5 |
| | vacuolised cells | 68.3 ± 12.4 | 40.3 ± 7.1* | 20.3 ± 11.1 | 20.6 ± 8.2 | 11.4 ± 6.3 | 39.1 ± 3.6** |
| $_{INF}MAC_{PBS}$ | monocyte-like cells | 94.7 ± 1.6 | 90.8 ± 7.1 | 2.8 ± 1.1 | 4.7 ± 3.1 | 2.5 ± 0.8 | 4.5 ± 3.1 |
| | vacuolised cells | 83.8 ± 10.4 | 76.4 ± 13.6 | 7.1 ± 3.8 | 8.2 ± 3.6 | 9.1 ± 5.4 | 15.4 ± 4.2 |
| $_{INF}MAC_{LPS}$ | monocyte-like cells | 84.5 ± 4.8 | 79.3 ± 10.8 | 9.1 ± 3.3 | 9.3 ± 4.9 | 6.4 ± 2.2 | 11.4 ± 5.9 |
| | vacuolised cells | 88.1 ± 3.1 | 83.3 ± 5.5 | 4.1 ± 1.6 | 2.2 ± 1.9 | 8.4 ± 1.1 | 10.4 ± 2.1 |
| Blood monocytes | | 90.4 ± 3.1 | 93.7 ± 6.2 | 3.7 ± 1.7 | 2.0 ± 1.2 | 5.9 ± 2.6 | 4.3 ± 2.5 |

Data are means in percentages, measured at 0 h and 6 h, and significant differences are marked by asterisks (*$P < 0.05$, **$P < 0.01$; Scheffe's method). The comparisons were made among 0 h samples relative to 6 h samples after cultivation *in vitro*

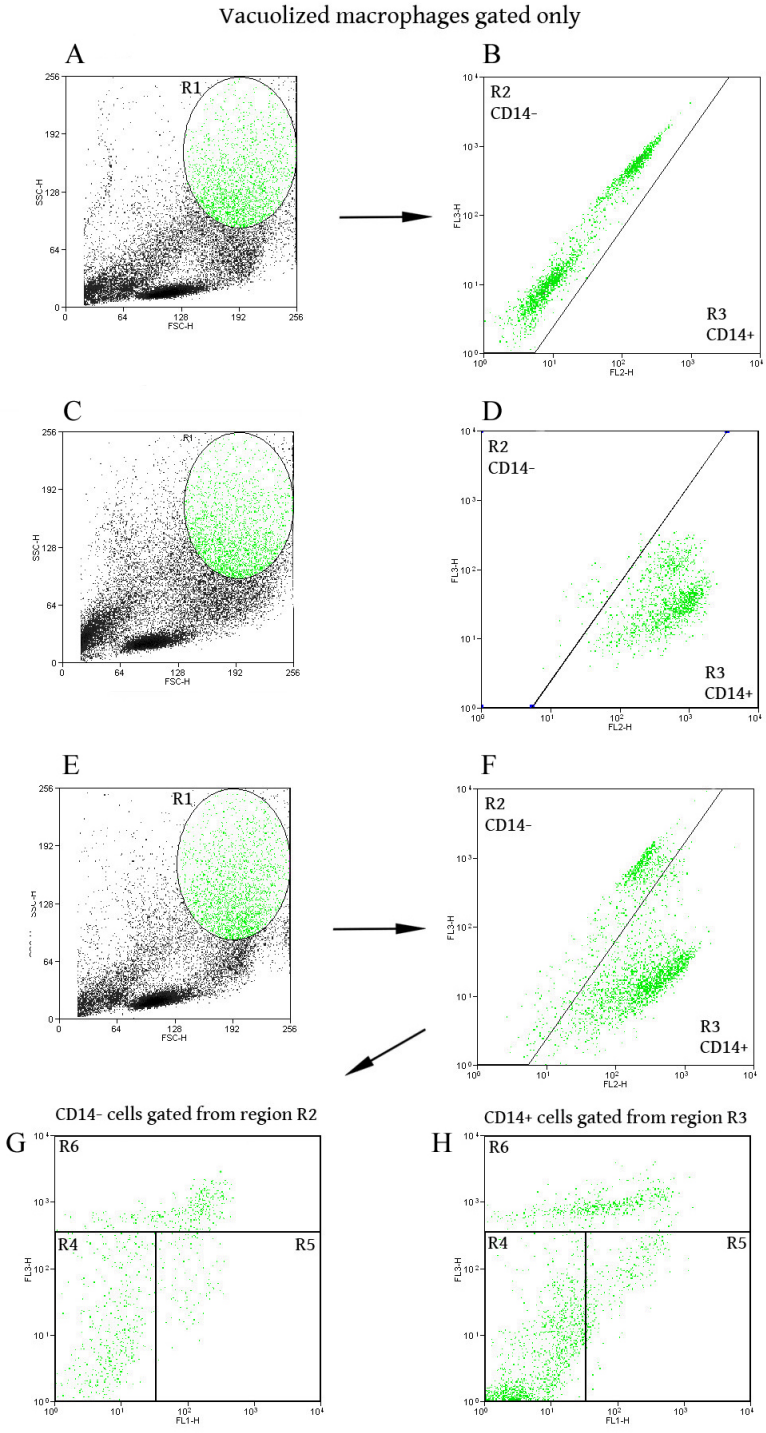


Figure 2. Compensation of AnnexinV-FITC, rPE and PI in flow cytometry and gating strategy for detection of CD14 expression, apoptosis and necrosis in vacuolised resident macrophages on representative dot plots. Dot plots **A** and **C** from control samples show the distribution of cells differentiated by their forward-scatter (FSC) and side-scatter (SSC) parameters, with the vacuolised $_{RES}$MAC populations highlighted in region R1. Dot plots **B** and **D** were gated from A and C dot plots. Dot plot B was gated from dot plot A, shows cells labelled with PI and with no rPE and gives a clean signal in region R2 on FL2 axis. Dot plot D was gated from C dot plot and shows cells labelled with no PI and with rPE and presents clean signal in region R3 on FL3 axis. Dot plot **E** from sample shows the distribution of cells differentiated by their forward-scatter (FSC) and side-scatter (SSC) parameters, with the vacuolised $_{RES}$MAC populations highlighted in region R1. Dot plot **F** was gated from dot plot E, shows cells labelled with PI and rPE and represents the distribution of CD14-positive (region R2 on FL2 axis) and CD14-negative macrophages (region R3 on FL3 axis). Dot plot **G** was gated from region R2 of dot plot F and shows CD14 negative cells labelled with FITC and PI → live (region R4 $CD14^-/AnnexinV^-/PI^-$), apoptotic (region R5 $CD14^-/AnnexinV^+/PI^-$), and necrotic cells (region R6 $CD14^-/AnnexinV^+/PI^+$). Dot plot **H** was gated from region R3 of dot plot F and shows CD14-positive cells labelled with FITC and PI → live (region R4 $CD14^+/AnnexinV^-/PI^-$), apoptotic (region R5 $CD14^+/AnnexinV^+/PI^-$), and necrotic cells (region R6 $CD14^+/AnnexinV^+/PI^+$)

monocytes (Table 2). Meanwhile, mostly living cells were observed in the population of $CD14^-$ macrophages and blood monocytes (Table 3).

## Living cells

In $CD14^+$ macrophages, the highest proportion of living cells was observed in monocyte-like $_{RES}$MAC and the smallest in vacuolised $_{INF}MAC_{LPS}$. *In vitro* cultivation led to significant increases in the proportions of living cells in all categories of $CD14^+$ macrophages (Table 2). In $CD14^-$ macrophages the highest proportion of living cells was observed in monocyte-like $_{INF}MAC_{PBS}$ and the smallest in vacuolised $_{RES}$MAC. Cultivation led to non-significant decreases in all categories of $CD14^-$ macrophages, and a significant decrease in vacuolised $_{RES}$MAC ($P < 0.05$) (Table 3).

### Apoptotic cells

In $CD14^+$ macrophages, the highest proportion of apoptotic cells was observed in vacuolised $_{INF}MAC_{LPS}$ and the smallest in monocyte-like $_{RES}MAC$. In contrast to living $CD14^+$ cells, *in vitro* cultivation led to significant decreases in the proportions of apoptotic cells in all categories of $CD14^+$ macrophages, and a non-significant decrease in vacuolised $_{INF}MAC_{LPS}$ (Table 2).

In $CD14^-$ macrophages, the highest proportion of apoptotic cells was observed in vacuolised $_{RES}MAC$ and the smallest in monocyte-like $_{RES}MAC$. *In vitro* cultivation led to non-significant increases in apoptotic cells, except for vacuolised $_{INF}MAC_{LPS}$ (Table 3).

### Necrotic cells

In $CD14^+$ macrophages, the highest proportion of necrotic cells was observed in vacuolised $_{INF}MAC_{LPS}$ and the smallest in monocyte-like $_{RES}MAC$. Compared to the apoptotic cells, *in vitro* cultivation led to non-significant decreases in the proportions of necrotic cells in all categories of $CD14^+$ macrophages (Table 2).

In $CD14^-$ macrophages, the highest proportion of necrotic cells was observed in vacuolised $_{RES}MAC$ and the smallest in monocyte-like $_{INF}MAC_{PBS}$ (Table 3). *In vitro* cultivation led to non-significant increases in the proportions of necrotic cells in all categories of $CD14^-$ macrophages, and a significant increase in vacuolised $_{RES}MAC$ ($P < 0.01$).

### Blood monocytes

In $CD14^+$ blood monocytes, we observed a significant decrease in live cells ($P < 0.01$) and a significant increase in apoptotic and necrotic cells ($P < 0.01$) after *in vitro* cultivation (Table 2).

In $CD14^-$ blood monocytes, we observed a non-significant increase in live cells and non-significant decrease in apoptotic and necrotic cells after *in vitro* cultivation (Table 3).

## DISCUSSION

This study evaluated the association of CD14 expression with cell viability in different types of bovine mammary gland macrophages obtained before and after an inflammatory response induced by CD14-dependent and -independent agents. To our knowledge, this is the first work comparing CD14 expression and cell death in macrophages in bovine veterinary medicine.

In fresh blood monocytes, we observed approximately 50% of cells to express CD14. This proportion is similar to that obtained for blood monocytes from dairy cows reported by Paape et al. (1996), who discovered a $CD14^+$ proportion of about 60% in the peripheral blood of lactating dairy cows and Kiku et al. (2010) who reported a rate of about 40% in cows postpartum.

We found that *in vitro* cultivation led to an approximately 50% decrease in CD14 expression in blood monocytes. This may correspond with the findings of Bosshart and Heinzelmann (2011), who observed that levels of CD14 on monocytic surfaces decreased on average by 25% after 4 h of incubation *ex vivo* and in the absence of inflammatory stimuli. The mechanism of decrease in CD14 expression in monocytes involves internalisation of membrane-bound CD14, followed by processing and secretion of soluble CD14 (Stelter 2000). A second possible mechanism for downregulating membrane-bound CD14 may be linked to the rapid recycling of CD14-TLR4-MD2 complexes between the plasma membrane and the Golgi apparatus (Latz et al. 2002).

In this study, decreased CD14 expression in bovine blood monocytes could be associated with an induction of apoptosis and necrosis similar to what has been reported in human blood monocytes (Heidenreich et al. 1997; Bosshart and Heinzelmann 2011). We exploited the discrimination of $CD14^+$ monocytes in dot plots to measure apoptosis and necrosis during cultivation. We found that the proportion of live cells in the population of $CD14^+$ monocytes was significantly decreased and the proportions of apoptotic and necrotic monocytes significantly increased. It is evident from these findings that the rapid decrease in CD14 expression in bovine blood monocytes is accompanied by decreased cell viability due to increases in apoptotic and necrotic $CD14^+$ cells.

Bovine mammary gland monocyte-like macrophages are derived from blood monocytes (Sladek and Rysanek 1999) and therefore they exhibit a similar expression of CD14. In contrast, in vacuolised macrophages we observed a higher expression of CD14. This may be related to their scavenger function, which is typical for some tissue-resident macrophages other than intesti-

nal and colonic macrophages (Smith et al. 2011). High CD14 expression has also been observed in alveolar-resident macrophages in the lungs (Jiang et al. 2003) and in mice peritoneal macrophages (Davicino et al. 2006). Alternatively, the positive effect on CD14 expression may be evoked by components of the mammary gland's alveolar and tissue microenvironment, as has been observed in mice lungs (Maus et al. 2001).

In this study, we observed a significant effect of LPS on CD14 expression in fresh mammary gland macrophages. This is in accordance with the observations of Paape et al. (1996), who reported a decreased proportion of $CD14^+$ mononuclear cells after intramammary injection of LPS. Similarly, Landmann et al. (1996) observed a reduction in the expression of CD14 mRNA and CD14 in monocytes and in monocyte-derived macrophages. Moreover, Lin et al. (2004) observed that LPS and *E. coli* significantly reduced the expression of CD14 in alveolar macrophages. Another explanation for this phenomenon is offered by the fact that CD14 is shed from the membrane into the extracellular space during the interaction of macrophages with LPS (Sugawara et al. 2000). Therefore, intramammary challenge with *E. coli* has been shown to induce significant increases in concentrations of soluble CD14 in infected quarters (Bannerman et al. 2004). The effects of LPS on CD14 expression are directly influenced by the degree of monocyte-to-macrophage differentiation (Landman et al. 1996) and mature macrophages with vacuolated cytoplasm express more CD14 (Dallard et al. 2009). Therefore, we observed a lower proportion of $CD14^+$ cells in monocyte-like $_{INF}MAC_{LPS}$ compared to vacuolised $_{INF}MAC_{LPS}$.

Cultivation *in vitro* led to a significant increase in CD14 expression in $_{INF}MAC_{LPS}$ in comparison to $_{INF}MAC_{PBS}$ and $_{RES}MAC$. This corresponds to data in the literature showing a stimulatory effect of LPS on CD14 expression in blood monocytes and the well-described biphasic pattern of LPS in tissue macrophages (Landmann et al. 1991; Chen et al. 1992; Hopkins et al. 1995). In a first phase, Landmann et al. (1996) found LPS to cause a weak reduction in CD14 transcription and CD14 expression after six to 15 h of LPS incubation in human monocytes as well as in macrophages. Similarly, incubation of rabbit alveolar macrophages for 24 h with LPS and *E. coli* has been shown to cause a significant decrease in CD14 expression (Lin et al. 2004). In the second phase, LPS caused a two-fold increase in CD14 RNA and CD14 expression after a two-day incubation (Landmann et al. 1996) or 48 h after intramammary LPS instillation (Sladek et al. 2002). The results of this study suggest that the expression of CD14 on the surface of macrophages from bovine mammary gland after instillation of LPS shows a biphasic pattern.

The increased CD14 expression in $_{INF}MAC_{PBS}$, $_{INF}MAC_{LPS}$ and $_{RES}MAC$ was accompanied by increasing proportions of apoptotic and necrotic cells during *in vitro* cultivation. We were interested in whether the increased expression of CD14 may be caused by apoptotic and necrotic cells. To address this question, we analysed apoptosis and necrosis in $CD14^+$ macrophages as well as in blood monocytes. The analysis of $CD14^+$ macrophages in flow cytometry showed that most of the $CD14^+$ macrophages are living cells, with the exception of the vacuolised $_{INF}MAC_{LPS}$, where live cells formed only one-third of the population. In contrast to blood monocytes, the proportion of live cells was significantly increased after *in vitro* cultivation in all $CD14^+$ macrophages. At the same time, the proportion of apoptotic and necrotic cells was significantly decreased in all $CD14^+$ macrophages. It is evident from these findings that the increase in CD14 expression in bovine mammary gland macrophages is accompanied by increased cell viability due to a decrease in the levels of apoptotic and necrotic cells.

In bovine mammary gland, apoptosis of $_{RES}MAC$ is associated with natural senescence (Sladek and Rysanek 2010). Therefore, a lower proportion of apoptotic cells is to be expected in monocyte-like $_{RES}MAC$ in comparison to vacuolised $_{RES}MAC$, because these are relatively young cells derived from monocytes and are rescued from early apoptotic death (Bellingan and Laurent 2008). In comparison to $_{RES}MAC$, apoptosis of $_{INF}MAC_{PBS}$ and $_{INF}MAC_{LPS}$ seems to be one of the critical events in the resolution of the inflammatory response (Sladek and Rysanek 2010), analogous to what is observed during resolution of acute lung injury (Janssen et al. 2011). Moreover, apoptosis and the phagocytosis of apoptotic macrophages were reported to be important in a murine model of pneumococcal infection (Marriott et al. 2006) and also appear to be involved in the intracellular killing of phagocytosed bacteria (Dockrell et al. 2001). In bovine mammary gland, the lower proportions of apoptosis in $_{INF}MAC_{PBS}$ and $_{INF}MAC_{LPS}$ are related to the initial phase of the inflammatory response (Sladek and Rysanek 2010). However, apoptosis was significantly delayed in $_{INF}MAC_{LPS}$ in comparison to $_{INF}MAC_{PBS}$ and also to $_{RES}MAC$ after *in vitro* incubation. This suggests

that LPS induces resistance to apoptosis and may promote the survival of these cells (Manna and Aggarwal 1999); further, the delayed induction of apoptosis in macrophages may limit tissue injury and contribute to the resolution of inflammation (Fischer et al. 2013). This anti-apoptotic response may correlate with the up-regulation of surface CD14 on $_{INF}MAC_{LPS}$, as has been noted in blood monocytes (Heidenreich et al. 1997).

In addition to apoptosis, we also observed necrosis in all types of macrophages after *in vitro* cultivation. In particular, high proportions of necrotic cells in vacuolised $_{RES}MAC$, $_{INF}MAC_{PBS}$ and $_{INF}MAC_{LPS}$ appear to be related to secondary necrosis of apoptotic macrophages. This has physiological significance during inflammation, since secondary necrosis can lead to the release of chemotactic peptides, which, in turn, may induce recruitment of monocytes into inflamed tissue. Moreover, proteins released from necrotic cells can opsonise apoptotic cells and thus facilitate their phagocytic uptake, which contributes to the resolution of inflammation (Blume et al. 2009; Blume et al. 2012).

It is therefore evident that there are significant differences among macrophages obtained from untreated and PBS- and LPS-treated mammary glands. Moreover, significant differences in CD14 expression, apoptosis and necrosis were also observed between monocyte-like and vacuolised macrophages. This is suggestive of different biological features in these cells and these potential differences should be taken into account in future studies of bovine mammary gland macrophages.

## CONCLUSION

Our experiments confirmed that the expression of CD14 in bovine mammary gland macrophages is not associated with cell death. In fact, higher CD14 expression corresponds to a higher proportion of live cells. Further studies will be needed in order to establish a cause and precise effect of CD14 expression on apoptosis, particularly *in situ* during the inflammatory response.

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