Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 57 (1): 60-65. April 2018
DOI: 10.5455/ajvs.295819

Macrophages Derived from Bovine Monocyte Subsets Differently Enhance the Vitality of Blood Neutrophils in Vitro

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Key words:
Bovine monocyte subsets; neutrophil; vitality; monocyte-derived macrophages; apoptosis

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ABSTRACT

Recent studies have shown that bovine blood monocytes represent a heterogeneous population of circulating cells with distinct phenotypic and functional properties. However, little is known about the heterogeneity of macrophages derived from different monocyte subsets. The current study aimed at investigating the functional heterogeneity of macrophages derived from the three bovine monocyte subsets regarding their capacity to influence the vitality of bovine blood neutrophils. Blood neutrophils were stimulated with culture supernatants from LPS-stimulated macrophages generated from different monocyte subsets. Vitality of stimulated neutrophils was analyzed by flow cytometry using the mitochondrial membrane potential probe JC-1. Similar to LPS, supernatant of stimulated monocyte-derived macrophages enhanced the life-span of neutrophils by reducing the percentages of apoptotic cells. The comparison between macrophages derived from different monocyte subsets showed that culture supernatants of macrophages derived from classical and intermediate monocytes induced the strongest anti-apoptotic effect on neutrophils. This suggests a role for macrophages derived from classical and intermediate monocytes rather than non-classical monocytes in prolonging the lifespan of neutrophils and therefore their contribution to innate immune defense during the acute phase of infection or inflammation.

1. INTRODUCTION

Recent studies have shown that bovine blood monocytes are a heterogeneous population of circulating cells with distinct phenotypic and functional properties (Hussen and Schuberth, 2017). Based on the cell surface expression of CD14 and CD16, bovine monocytes are subdivided into classical (cM, $CD14^{++}$ $CD16^{-}$), intermediate (intM, $CD14^{++}$ $CD16^{+}$) and non-classical (ncM, $CD14^{-}$ $CD16^{++}$) monocyte subsets (Hussen et al., 2013, Hussen et al., 2016, Hussen and Schuberth, 2017, Koy et al., 2013, Pomeroy et al., 2017).

After leaving the blood stream, monocytes differentiate into tissue monocyte-derived macrophages, that interact with neutrophils during all phases of inflammatory response (Holzmuller et al., 2016, Pomeroy et al., 2016, Jin and Kruth, 2016, Singleton et al., 2016, Baquero and Plattner, 2016, Summerfield et al., 2015). Gene expression profiles of macrophages generated from bovine monocyte subsets in vitro indicate a heterogenic differentiation potential of bovine monocyte subsets into distinct functional macrophage subtypes (Schütz et al., 2013).

Neutrophils are key innate immune cells rapidly recruited to sites of infection, where they phagocytose and kill invading microorganisms. Under normal conditions, circulating mature neutrophils have the shortest lifespan among leukocytes in the circulation (Fox et al., 2010, Kumar and Sharma, 2010, Witko-Sarsat et al., 2000). During the early stages of infection, the life span of neutrophils is prolonged to enhance anti-microbial functions of these phagocytic cells (Haslett et al., 1991, Lin et al., 2017). Several macrophage mediators, including G-CSF, GM-CSF,

IL-8, IL-1b and TNFa have the ability to delay neutrophil apoptosis ensuring better functional capacity of neutrophils during the acute phase of infection (McCracken and Allen, 2014, Kobayashi et al., 2005, Geering et al., 2013, Kennedy and DeLeo, 2009).

The current study aimed at investigating the functional heterogeneity of macrophages derived from the three bovine monocyte subsets regarding their capacity to influence the vitality of bovine blood neutrophils.

2. MATERIAL AND METHODS

2.1 Animals and blood collection

All procedures involving animals were carried out in accordance with German legislation on animal welfare. The commissioner for animal welfare at the University of Veterinary Medicine, Hannover, Germany, approved the mentioned procedures. Blood was obtained from healthy Holstein Friesian cows (n= 7 cows), housed in the Clinic for Cattle (University of Veterinary Medicine, Hannover, Germany) and was drawn into heparinized vacutainer tubes from the *vena jugularis externa*.

2.2 Sorting of bovine monocyte subsets

Blood was layered on Ficoll-Isopaque (PAA, Pasching, Austria) and centrifuged at 10°C for 30 min at 1000 x *g*. The interphase containing mononuclear cells was washed three times in PBS (500 x *g*, 200 x *g* and 100 x *g*, 4°C). Bovine monocyte subsets were separated as described earlier (Hussen et al., 2013). Briefly, separated mononuclear cells (MNC) were labeled with a FITC-conjugated CD16 specific antibody (clone KD1, AbD Serotec) for 15 min and subsequently with anti-FITC MultiSort MicroBeads (Anti-FITC MultiSort Kit; Miltenyi Biotec) to separate MNC into a CD16-positive fraction (containing intM and ncM) and a CD16-negative fraction (containing cM and lymphocytes). To release the anti-FITC MultiSort MicroBeads from the selected CD16-positivefraction, cells were incubated with the MultiSort release reagent (included in the anti-FITC MultiSort Kit) for 10 min. CD16-positive cells (positively selected) and CD16-negative cells (in the flow throw) were subsequently incubated with anti-CD14 MicroBeads (Miltenyi Biotec) and separated according to the manufacturer's instructions into 3 monocyte subsets. CD14-positive cells were positively selected from the CD16-negative fraction (cM) and the CD16-positive fraction (intM). Remaining cells in the flow throw of the CD16-positive fraction presented ncM being CD14-negative and CD16-positive. All steps of monocyte subset isolation were performed at 4°C. After each labeling step, the cells were washed with PBS-EDTA (300 x g, 10 min). Negatively and positively selected cells were checked flow cytometrically for their purity after labeling the cells with a PE-conjugated mouse anti bovine CD14 monoclonal antibody (clone TÜK4, AbD Serotec) (Fig.1). Cell vitality as measured after adding propidium iodide (2 µg/ml) was above 92% for all subsets.

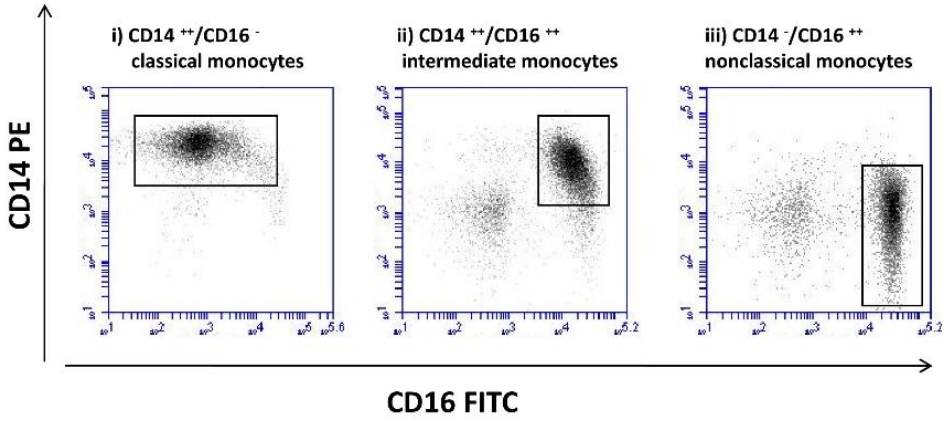

Figure 1. Purification of bovine monocyte subsets. Three-color immunofluorescence of MACS-separated bovine classical (CD14 ++ CD16-) (i), intermediate (CD14 ++ CD16+) (ii) and non-classical monocytes (CD14 - CD16++) (iii) after labelling with mAbs to CD172a, CD14 and CD16.

2.3 In vitro generation and stimulation of monocytes-derived macrophages

Macrophages were generated in vitro from separated blood monocyte subsets according to an earlier method (Castano et al., 2011). Briefly, MACS-separated monocyte subsets were suspended in RPMI medium (PAA, Pasching, Austria) supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin at $2x10^5$ cells per ml. Cells were cultivated in 24 well plates (Costar, Cambridge, MA) at $2x10^5$ cells per well for 4 days (37°C, 5% CO_2). After 4 days of cultivation fresh pre-warmed medium containing 1µg/ml LPS was added to the cells and plates were incubated for further 24 h (37°C, 5% CO_2). After incubation, culture supernatants were harvested and used for stimulation of neutrophils (PMN).

2.4 Separation and stimulation of blood neutrophils

The PMN-erythrocyte containing phase, after harvesting the MNC-containing interphase, was suspended in distilled water for 20 sec and tonicity was restored by adding double concentrated PBS. This was repeated (usually twice) until complete erythrolysis. PMN were finally suspended in RPMI medium at 5 x 10^6 cells/ml. Separated bovine PMN ($5x10^5$/well) were incubated (4 h at 37°C) in 96-well round-bottomed culture plates with culture supernatants of LPS-stimulated macrophages derived from MACS-separated cM, intM or ncM. Parallel set ups were stimulated with LPS (1µg/ml) or were left without stimulation.

2.5 Analysis of apoptosis in stimulated PMN

Apoptosis in PMN was analysed according to an earlier study (Troiano et al., 2007). Briefly, In vitro stimulated PMNs were centrifuged at 250 x g for (4 min, 20°C) and the supernatant was discarded. Cells were washed once in PBS (100 µl, 250 x g, 20°C, 4 min). JC-1 solution (100 µL, 7 µmol/L) was added to the cell pellet and incubated for 15 min at 37°C. After two washing steps in PBS, cells were suspended in 200 µL sterile-filtered PBS containing 2 µg/ml To-Pro-3, transferred to flow cytometer tubes and acquired with the Accuri flow cytometer. For all set ups, at least 10^4 events were acquired and the percentages of apoptotic and viable PMN were calculated and presented as Mean ± SEM.

3. Statistical analyses

Statistical analysis was performed using the statistical software Prism (GraphPad software). Results are expressed as means ± S.E. of the mean (SEM). Differences between means were tested with the one-factorial analysis of variance (ANOVA). Results were considered statistically significant at a p-value of less than 0.05.

4. RESULTS

4.1 Flow cytometric analysis of neutrophil (PMN) apoptosis

After staining with JC-1 and To-Pro-3, bovine PMN were analyzed by flow cytometry (Fig. 2). PMN were firstly identified according to their forward (FSC) and sideward (SSC) scattering characteristics. Apoptotic cells among To-Pro-3-negative cells (viable cells) were determined after staining with the mitochondrial membrane potential probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide, Molecular Probes). Viable cells incorporate the lipophilic and positively charged JC-1-monomers into the negatively charged mitochondria-matrix and form red fluorescing aggregates (Troiano et al., 2007). In a FL1 versus FL4 dot plot, apoptotic PMN, which were defined as JC1-positive and To-Pro-3 negative cells, displayed a higher green (FL1-fluorescence) compared to non-apoptotic, viable cells. Necrotic PMN were defined as To-Pro-3-positive cells. Viable non-apoptotic PMN were defined as JC1-negative and To-Pro-3 negative cells.

4.1 Supernatants from LPS-stimulated macrophages derived from bovine monocyte subsets differentially affect apoptosis of bovine PMN

Incubation of PMNs for 4 h in culture medium without stimulation resulted in over 50 % apoptosis rate (53 % ± 5) of total PMN. This percentage was reduced when PMN were stimulated with LPS (42 % ± 3). However, stimulation of PMN with supernatants collected from LPS-stimulated macrophages derived from bovine monocyte subsets induced higher percentages of viable cells and lower rates of apoptosis. Comparison of macrophages derived from the three monocyte subsets revealed a higher anti-apoptotic effect through stimulation with supernatants from macrophages derived from classical (cM; 28 % ± 1) and intermediate (intM; 27 % ± 3) monocytes in comparison to macrophages derived from non-classical monocytes (ncM; 34 % ± 1) (Fig. 3).

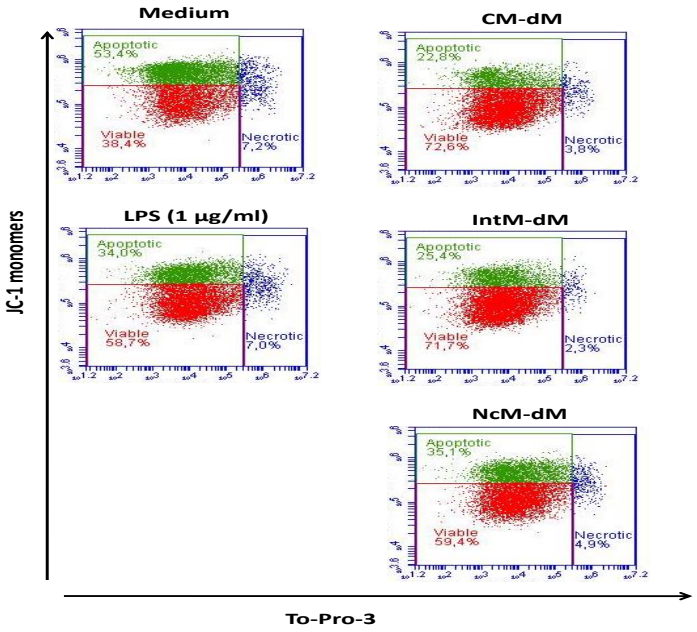

Figure 2. Flow cytometric analysis of apoptosis and necrosis in bovine blood neutrophils (PMN)
Separated bovine PMN ($5x10^5$, n= 7 animals) were incubated without stimulation (medium), stimulated with LPS (1µg/ml) or incubated with culture supernatants of LPS-stimulated macrophages derived from MACS-separated classical (classical monocytes-derived macrophages; CM-dM), intermediate (classical monocytes-derived macrophages; IntM-dM) or non-classical (classical monocytes-derived macrophages; ncM-dM) bovine monocyte subsets for 4 h at 37°. Stimulated PMN were stained with To-Pro-3 and JC-1 and stained cells were analyzed by flow cytometry. PMN were firstly identified according to their forward scatter (FSC) and sideward scatter (SSC) characteristics. In a FL1/FL4 dot plot, apoptotic PMN were defined as JC1-positive and To-Pro-3 negative cells. Necrotic PMN were defined as To-Pro-3-positive cells. Viable non-apoptotic PMN were defined as JC1-negative and To-Pro-3 negative cells. Representative flow cytometric analysis of one animal.

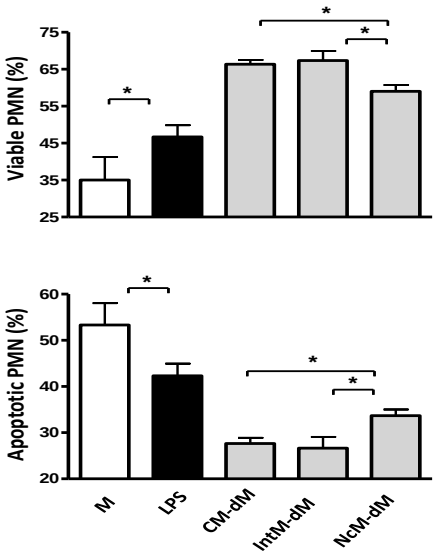

Figure 3. Impact of supernatants from LPS-stimulated macrophages derived from different bovine monocyte subsets on vitality of neutrophils. Separated bovine PMN (n= 7 animals) were incubated without stimulation (medium), stimulated with LPS (1µg/ml) or incubated with culture supernatants of LPS-stimulated macrophages derived from MACS-separated (classical monocytes-derived macrophages; CM-dM), intermediate (classical monocytes-derived macrophages; IntM-dM) or non-classical (classical monocytes-derived macrophages; ncM-dM) bovine monocyte subsets for 4 h at 37°. Stimulated PMN were stained with To-Pro-3 and JC-1 and stained cells were analyzed by flow cytometry. For all set ups, the percentages of apoptotic and viable PMN were calculated and presented as Mean ± SEM. Effects were considered significant when $p< 0,05$.

5. DISCUSSION

Based on their cell surface expression of CD14 and CD16, bovine peripheral blood monocytes have been recently grouped into classical (cM, $CD14^{++}$ $CD16^{-}$), intermediate (intM, $CD14^{++}$ $CD16^{+}$) and non-classical (ncM, $CD14^{-}$ $CD16^{++}$) subsets (Hussen et al., 2013, Hussen and Schuberth, 2017), which have been shown to be heterogenic in term of phenotype, function and their relevance to bovine infectious diseases (Hussen et al., 2013, Hussen et al., 2016, Hussen and Schuberth, 2017, Koy et al., 2013, Pomeroy et al., 2017).

Polymorphonuclear neutrophils (PMN) are innate immune cells that communicate with monocytes and monocyte-derived macrophages during all phases of infection and inflammation (Witko-Sarsat et al., 2000, Amulic et al., 2012, Hussen et al., 2016). Under normal conditions, circulating PMN have a short half-life and they rapidly undergo programmed apoptotic cell death. During the early stages of infection, the life-span of PMN is prolonged to enhance the anti-microbial function of these phagocytic cells (Haslett et al., 1991, Lin et al., 2017).

Lipopolysaccharides (LPS) belongs to the most investigated microbial patterns that have been shown to prolong PMN life span by inhibition of apoptosis (McCracken and Allen, 2014). Although our results agree with earlier studies regarding an anti-apoptotic effect of LPS on PMN, however in the current study LPS stimulation did not lead to a larger fraction of cells undergoing necrosis as reported earlier (Turina et al., 2005), which may be due to different stimulation periods.

Bovine monocyte subsets have shown some variation in their differentiation potential into phenotypically and functionally distinct macrophage subtypes (Schütz et al., 2013). In the current study, stimulation of PMN with supernatant collected from LPS-stimulated macrophages derived from different bovine monocyte subsets enhanced the life-span of PMN by reducing the percentages of apoptotic cells. This indicates the secretion of macrophages anti-apoptotic factors that prolong the life span of PMN. The comparison between macrophages derived from different bovine monocyte subsets showed, that this anti-apoptotic effect is mainly mediated by macrophages derived from cM and intM subsets. This seems to be in line with the described pro-inflammatory nature of cM and intM rather than ncM (Hussen et al., 2013, Hussen and Schuberth, 2017) as well as with the highest expression of *TNF*, *IL1*, *iNOS* and *CXCL8* in LPS-stimulated macrophages derived from bovine cM and intM in comparison to ncM (Schütz et al., 2013). This suggests a role for macrophages derived from cM and ncM rather than the ncM subset in prolonging the lifespan of neutrophils and therefore their contribution to innate immune defense during the acute phase of infection, where extending the lifespan of neutrophils has been shown to be critical for efficient destruction of pathogens (Nathan, 2006). On the other hand, the lowest anti-apoptotic effect through macrophages derived from ncM indicates a role for this subset in the late stages of infection or inflammation, where neutrophil apoptosis contributes to resolution of inflammation (Fox et al., 2010, Martin et al., 2015).

Several macrophage mediators including G-CSF, GM-CSF, IL-8, IL-1β and TNFα have been shown to temporarily delay neutrophil apoptosis as a mean to ensure a better functional capacity of neutrophils during the acute phase of infection (McCracken and Allen, 2014, Kobayashi et al., 2005, Geering et al., 2013, Kennedy and DeLeo, 2009). However, specific mediators in supernatants from bovine monocyte-derived macrophages that specifically mediate this anti-apoptotic effect on bovine PMN are still to be determined.

In conclusion, macrophages derived from different bovine blood monocyte subsets differ in their ability to influence neutrophil vitality in vitro.

6. Acknowledgements

I highly acknowledge the support in data analysis I received from Prof. Hans-Joachim Schuberth at Immunology Unit, University of Veterinary Medicine Hannover, Hannover, Germany. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

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