Research paper

Optimized *in vitro* isolation of different subpopulation of immune cells from peripheral blood and comparative techniques for generation of monocyte-derived macrophages in small ruminants

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Abstract

Peripheral blood from healthy sheep (n=3) and goats (n=3) were employed to establish an efficient method for simultaneous isolation of peripheral blood mononuclear cells (PBMCs) and neutrophils and to standardize protocols for monocyte purification and generation of monocyte-derived macrophages (MDMs). In both species, a significantly enriched population of PBMCs, with higher purity and number of cells determined by flow cytometry, was achieved when processing through a density gradient a mixture of buffy-coat and red blood cell layer (RBC) in comparison to the use of just the buffy-coat ($p < 0.05$). Neutrophils could be subsequently isolated from the layer, located underneath PBMCs fraction with significant higher purity rates, higher than 85% determined by flow cytometry, than those obtained with protocols without density gradients (< 60%) ($p < 0.05$). This technique would allow the isolation of both cell populations from the same sample of blood. A pure cell population of monocytes, $CD14^+$ cells, was purified from PBMCs when using immunomagnetic columns, which allow for 17% (nº monocytes/nº PBMCs) of yield and high percentages of expression of $CD14^+$ (88%), $MHC\text{-}II^+$ (91.5%) and $CD11b^+$ (94%) established by flow cytometry. On the other hand, the classical and non-expensive purification of monocytes from PBMCs based on the adherence capacity of the former, allowed significantly lower yield of monocytes (4.6%), with percentages of surface markers expression that dropped to 35%, 65% and 55%, respectively ($p < 0.001$), suggesting the isolation of a mixed population of cells. The addition of GM-CSF to the culture, at concentration from 25 to 125 ng/ml, enhanced proportionally the number of MDMs generated compared to the absence of supplementation or the use of autologous serum from 5% to 20%. However, purification of monocytes through the adherence method achieved higher yields of MDMs than those isolated through immunomagnetic columns in both species ($p < 0.001$). Under the conditions of this study, the use of centrifugation in density gradients allow for the simultaneous purification of PBMCs and neutrophils, with high purity of both populations, from the same sample of blood. The isolation of monocytes could be subsequently achieved through two different methods, i.e. based on immunomagnetic columns or adherence. The preference

between both methods would depend on the necessities of the experiment, the initial sample with high purity of monocytes or a final population of MDMs required.

Keywords: PBMCs; neutrophils; monocytes; monocyte-derived macrophages; sheep; goats.

Abbreviations: AICc, Akaike's Information Criterion; BSA, bovine serum albumin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; GLMs, generalized linear models; GM-CSF, granulocyte macrophage colony-stimulating factor; LM, linear model; MDMs, monocyte-derived macrophages; PBMCs, peripheral blood mononuclear cells; RBC, red blood cell; SCC, side scatter.

1. Introduction

Macrophages and neutrophils play a crucial role in the innate immune response defending the host from pathogens and maintaining homeostasis, although they also take part in the adaptive immune response priming other immune host cells for enhanced microbicidal activity (Gordon and Taylor, 2005; Borregaard et al., 2007). Due to their biological function, their study is the key for a greater understanding of the host-pathogen interaction, particularly in those caused by intracellular pathogens. Cell culture allows the study of the response of these cells after their contact with the pathogen (expression and production of cytokines, phagocytosis or microbicidal activity) (Borrmann et al., 2011; Elmowalid et al., 2013). In human research, there is a well standardized methodology for the *in vitro* culture of these cells. In contrast, in veterinary research, and especially in small ruminants, there is lack of a common agreement with respect to the development of an effective method for the isolation and culture of monocytes, macrophages and neutrophils.

Since the isolation of mature macrophages from tissues is labour-intensive and ineffective, the generation of monocyte-derived macrophages (MDMs) from blood monocytes is the most feasible approach to obtain these immune cells (Daigneault et al., 2010). Blood monocytes are present within the mononuclear cells fraction of the peripheral blood. To isolate

this fraction, the use of density gradients, including a variety of chemical compounds such as Percoll®, Histopaque®, Ficoll-Paque™ or Lymphoprep™ is the most well-known and employed method in ruminants following different protocols (Olivier et al., 2001; Keane et al., 2002; Souza, 2015). Commonly, once peripheral blood mononuclear cells (PBMCs) are isolated, monocytes are purified based on their property of adhesion on plastic surfaces and subsequent elimination of non-adherent cells by washing culture plates (Olivier et al., 2001; Berg et al., 2013; Elmowalid et al., 2013). However, the use of immunomagnetic columns and specific anti-human antibodies coupled with magnetic beads has become a common procedure in human research for a fast and efficient isolation of various cell populations such as peripheral blood monocytes (Nakazawa et al., 2016; Nielsen et al., 2020). In this sense, the proved existence of cross-reaction of these antibodies with antigens of ruminant monocytes has also allowed its use with apparently optimal results in cattle and sheep (Budhia et al., 2006; Machugh et al., 2012; Souza, 2015).

Once purified, *in vitro* culture of monocytes allows the obtaining of MDMs through a maturation process that depends on suitable conditions of culture and permits their use as a model for the study of macrophages (Daigneault et al., 2010; Elmowalid, 2012). In this sense, the influence of serum and/or different growth factors on the maturation and polarization of MDMs are well-established in human research (Eske et al., 2009; Ambarus et al., 2012; Vogel et al., 2014). However, this maturation has been carried out in ruminants using specific culture media supplemented with fetal bovine serum (FBS) or autologous serum (Werling et al., 2004; Gollnick et al., 2007; Elmowalid, 2012) together with the addition of granulocyte macrophage colony-stimulating factor (GM-CSF) to supplemented media (Budhia et al., 2006; Souza, 2015; Pomeroy et al., 2016; García-Sánchez et al., 2019). Nevertheless, the optimal concentrations of GM-CSF are unclear and vary from 4 ng/ml to 100 ng/ml in these studies. All these studies state that MDMs are suitable for subsequent functional studies, however, there are no evidences that determine which of these culture conditions are the most appropriate to gain the largest number of MDMs, especially in sheep and goats.

Neutrophils are also an important cell population that actively participates in the host immune response. As in the case of MDMs, their study under *in vitro* conditions allows the knowledge about mechanisms of the host immune response under tightly controlled conditions (Borregaard, 2010). While in human research there are standardized protocols for the isolation of neutrophils (Siemsen et al., 2014), different methods have been proposed for their isolation from peripheral blood in ruminants, most of them based on the direct lysis of erythrocytes (Paltrinieri et al., 2000; Hellenbrand et al., 2013; Jimbo et al., 2017) and few of them based on the centrifugation of blood with density gradients compounds (Silva et al., 2014; Cacciotto et al., 2016). The purity and quality of the isolated neutrophils in ruminants are generally assessed by direct microscopic visual evaluation and/or analysis of the size and granularity of the cells through flow cytometry (Carretta et al., 2013; Zoldan et al., 2014; Kamoshida et al., 2015), obtaining similar and optimum results despite the wide range of available protocols.

Despite the great variety of protocols for the isolation of neutrophils and monocytes employed in different ruminant species (mainly cattle), their use in small ruminants (sheep and goats) is scarce and the fewer published studies show important differences on the methodology (Elmowalid, 2012; Siemsen et al., 2014; Silva et al., 2014). In this sense, an agreed protocol for their isolation would help to make comparable all the studies carried out in these ruminant species. Thus, due to the importance of these cell populations, the main objective of this study is to establish a suitable and effective procedure for PBMCs and neutrophil isolation from the same blood sample in sheep and goats as well as establish the best procedure for the purification of monocytes from PBMCs and the subsequent generation of MDMs exploring and comparing different technical protocols.

2. Material and Methods

2.1. Animals

This study was carried out using healthy, non-pregnant, one year old sheep (n = 3) and goats (n = 3) of assaf and murciano-granadina breeds respectively, belonging to the

experimental herd of the Instituto de Ganadería de Montaña (ULE-CSIC) in Grulleros, León, Spain. All the procedures have been designed according to European (86/609) and Spanish laws (R.D. 223/1988, R.D. 1021/2005 and R.D. 53/2013). Handling and sampling procedures were minimized in order to reduce stress and health risks for the animals and staff involved. A blood sample (200 ml) was collected from each animal twice a month to reduce stress and allow their recovery. Then, three technical replicates were performed from each animal to carry out all the different procedures.

2.2. PBMCs isolation

Two different protocols for the isolation of PBMCs were evaluated (Fig. 1):

2.2.1. Isolation from buffy-coat

Whole blood samples were collected by jugular venipuncture using 10 ml BD Vacutainer™ tubes with lithium heparin as anticoagulant and PBMCs were isolated from the buffy-coat fraction as previously described Chan et al. (2002) with few modifications (Fig. 1). Briefly, whole blood was centrifuged at 650 x *g* for 30 minutes at room temperature with no stop or acceleration. The obtained buffy-coat was collected and topped up to 15 ml with PBS/EDTA (2 mM EDTA, pH. 8) to be layered upon 15 ml of Lymphoprep™ (STEMCELL Techonolgies®, Grenoble, France) and centrifuged at 800 x *g* for 30 minutes under the same conditions mentioned above. The interphase of mononuclear cells isolated from PBMCs and located between plasma and Lymphoprep™ fractions was aspirated and washed with 30 ml PBS/EDTA by centrifugation at 300 x *g* for 10 minutes at 4°C. Later on, the cell pellet was resuspended in 3 ml of 0.03% of sodium bicarbonate for the lysis of erythrocytes, shaken for 10 seconds and isotonicity was restored adding 40 ml of PBS/EDTA and centrifuged at 300 x g for 10 minutes. Then, the cell pellet was washed again with 20 ml of PBS/EDTA and resuspended in 15 ml of RPMI1640 medium+GlutaMAX™ with phenol red (Gibco, ThermoFisher Scientific®) supplemented with 10% of heat-inactivated FBS (Gibco, ThermoFisher Scientific®),

1% of penicillin/streptomycin/amphotericin (Santa Cruz Biotechnology®) and 50 µM of 2-β-Mercaptoethanol (Gibco, ThermoFisher Scientific®).

2.2.2. Isolation from buffy-coat and red blood cell layer

Blood samples were collected as explained above (2.2.1. section) and were diluted at a 1:3 proportion in PBS/EDTA (Fig. 1). Diluted blood was centrifuged at 650 x *g* for 30 minutes at room temperature with no stop or acceleration and the buffy-coat plus the layer of red blood cells (RBC) layer located immediately below were collected, topped up to 15 ml with PBS/EDTA and layered upon Lymphoprep™ (STEMCELL Techonolgies®, Grenoble, France). Afterwards, the PBMCs interphase was collected and resuspended as previously mentioned above (2.2.1.).

2.2.3. Cell counting and flow cytometry analysis

In order to compare both methods, the number of PBMCs was counted using a Neubauer chamber. Besides, their size and complexity were characterized through the analysis of 10.000 events using a flow cytometer (MACSQuant, MiltenyiBiotec®) and the percentage of PBMCs was estimated using side scatter (size) versus forward scatter (granularity) plots (SCC:FSC), gating the characteristic location of this populations in order to calculate the percentage of purity using the MACSQuantify10 Software™. (MiltenyiBiotec®).

2.3. Monocyte purification

Two purification techniques for monocyte purification from PBMCs were assessed using previously isolated PBMCs from the buffy-coat plus RBC layer (method explained at 2.2.2. section) due to the favorable results obtained during PBMCs isolation.

2.3.1. Purification of monocytes by the adherence method

Monocytes were purified from PBMCs as previously describe Elnaggar et al. (2016) for sheep and goats with few modifications. A total of $1.5x10^7$ PBMCs per well were seeded on a

six-well culture plate (TPP®, Sigma-Aldrich) in a final volume of 3 ml RPMI1640 supplemented medium mentioned above and were incubated for 3 hours at 37°C and 5% CO_2 in a humidified incubator. Subsequently, non-adherent cells were carefully removed with warm PBS. In order to estimate the number of purified monocytes, plates were placed on ice for 15-20 minutes with 3 ml of cold PBS/EDTA and were slightly scraped and recovered by gentle pipetting.

2.3.2. Purification of monocytes by immunomagnetic columns

As previously described Budhia et al. (2006) for sheep, a $CD14^+$ cell selection was performed from PBMCs using MACS LS columns (MiltenyiBiotec®) according to the manufacturer's instructions. Briefly, a total of $2x10^7$ PBMCs were pelleted and resuspended in 40 µl solution of microbeads conjugated to monoclonal anti-human CD14 antibody (MiltenyiBiotec®), diluted in 160 µl of PBS/EDTA supplemented with 0.05% of bovine serum albumin (BSA) and incubated during 15 minutes at 4°C. Afterwards, PBMCs were washed with 4 ml of PBS/EDTA and resuspended in 500 µl of PBS/EDTA/BSA. PBMCs were then loaded onto LS separation column placed in the MACS magnet and a positive selection of $CD14^+$cells was performed, with $CD14^+$ cells retained in the column, while the negative cells passed through it. Finally, the column was removed from the magnetic field and 5 ml of RPMI 1640 supplemented medium was passed through the column, with the aid of a plunger, to wash, and purify the $CD14^+$ cells.

2.3.3. Monocyte yield and phenotypic analysis

The quantity and phenotypic characteristics of purified monocytes obtained by both purification techniques were compared. In order to calculate the yield of monocytes obtained though adherence method and immunomagnetic columns, recovered adherent cells and $CD14^+$ cells, respectively were counted in a Neubauer chamber. The yield of monocytes of each purification techniques was calculated as the percentage of purified monocytes (number of monocytes/ number of initial PBMC*100) in both animal species.

Afterwards, the phenotype of the monocytes obtained through both purification techniques was studied using flow cytometry analysis. Briefly, besides the analysis of the size and complexity of the cells, monocytes were labeled using monoclonal antibodies anti-bovine CD14 (clone CC-G33, MCA2678, Bio-Rad®) and anti-sheep MHC-II (clone 37.68, MCA2226, Bio-Rad®) at a 1:100 dilution, and anti-bovine CD11b (clone CC126, MCA1425, Bio-Rad®) at a 1:150 dilution for 1 hour at 4°C. Then, cells were washed with PBS and incubated with Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody at a 1:50 dilution (F0313, Dako®) for one 1 hour in darkness at 4°C. Finally, cells were washed again and fixed with 1% BD CellFIX™. Sample acquisition of 10.000 events was performed using a flow cytometer (MACSQuant, MiltenyiBiotec®) where events were gated during the acquisition analysis (Supplementary Figure 1) to discard the presence of air and doublets. Analysis of data was performed using the MACSQuantify10 Software™ (MiltenyiBiotec®). Results were expressed as percentage of positive cells and mean fluorescence intensity for each cell phenotype (Corripio-Miyar et al., 2015).

2.4. Generation of MDMs

2.4.1. In vitro culture conditions of monocytes

Generation of MDMs was carried out by two different methods by means adding different concentrations of ovine GM-CSF (RP1190V-100, KingFisher Biotech®, INC) or autologous serum to the supplemented RPMI 1640 culture media in monocytes purified through the adherence method as explained in 2.3.1. section. GM-CSF concentrations of 0 ng/ml, 25 ng/ml, 60 ng/ml and 125 ng/ml as well as autologous serum at 5%, 10% and 20% concentrations were employed. Autologous serum was obtained from the same animals used for monocyte isolation, collecting blood by jugular venipuncture into 10 ml BD Vacutainer™ tubes without anticoagulant and allowed to clot overnight. Afterwards, samples were centrifuged at 430 x *g*, 15 minutes at 4ºC and serum was collected and heat-inactivated at 56ºC for 30 minutes.

Finally, serum was filter-sterilized through 0.2 µm filter (10462200, Biotech®) and aliquoted until use.

Supplemented RPMI 1640 media cell culture with GM-CSF or autologous serum was added to each well and changed the third day of culture with fresh media. Each concentration was studied by duplicate. At the seventh day of culture, plates with MDMs were carefully washed twice with 3 ml of warm PBS and harvested by adding and 3 ml of cold PBS/EDTA and placed on ice for 20 minutes. MDMs were then slightly scraped and recovered in 5 ml of RPMI1640 supplemented medium.

2.4.2. Purification conditions of monocytes

To further analyze whether the purification of monocytes through adherence method or immunomagnetic columns could influence the generation of MDMs, in terms of the total number of recovered MDMs and their phenotype, purified monocytes by means of both purification techniques and generated MDMs after 7 days of supplementation with 60 ng/ml of GM-CSF were scraped as mentioned above and recovered in 5 ml of RPMI1640 supplemented medium until use.

2.4.3. MDM yield and phenotypic analysis

The morphological features of MDMs obtained in sections 2.4.1. and 2.4.2. (changes on the size, shape and granularity as well as dimension of cytoplasm) were daily assessed by the examination of the culture plates under inverted microscope (LEITZ DM IL®, Leica), from the first to the seventh day of culture.

The number of recovered MDMs with different *in vitro* conditions and the yield of recovered MDMs with different purification techniques was counted with a Neubauer chamber and were estimated as the total number of recovered MDMs.

To characterize the phenotype of these MDMs, size, complexity and the expression of different surface markers were analyzed by flow cytometry (MACSQuant, MiltenyiBiotec®). MDMs were labeled using the same antibodies and methodology explained in 2.3.3. section.

The percentage of positive cells and fluorescence intensity was recorded and compared between both purification techniques but also between monocytes and MDMs obtained by both purification techniques. Thus, differences between cell type, purification technique and animal species were considered as variables in further analysis.

2.5. Neutrophils isolation from peripheral blood

Three different methods for the isolation of neutrophils from peripheral blood were evaluated.

2.5.1. Isolation of neutrophils without density gradients

This method is based on previous works carried out in sheep and cattle with slight modifications (Paltrinieri et al., 2000; Hellebrand et al., 2013). Heparinized blood were collected by jugular venipuncture and centrifuged at 400 x *g* for 20 minutes. The RBC layer just below the buffy-coat, where the neutrophil fraction is present, was collected and diluted in distilled water for erythrocytes lysis in a dilution of 1:3 by gently shaking for 10 seconds. Then, neutrophils were diluted in PBS/EDTA to restore isotonicity, pelleted at 300 x *g* for 10 minutes and washed with PBS/EDTA four times. Finally, pelleted neutrophils were resuspended in RPMI1640 medium (Gibco, ThermoFisher Scientific®).

2.5.2. Isolation of neutrophils using Percoll®

This method is based on previous works conducted in small ruminants with few modifications (Woldehiwet et al., 2003; Siemsen et al., 2014). Heparinized blood samples were centrifuged and the RBC layer was collected as mentioned above. Erythrocytes were lysed using 20 ml distilled water, inverting tubes for 20 seconds. The isotonicity was restored by the addition of 20 ml of PBS/EDTA and tubes were centrifuged at 250 x *g* for 5 minutes at room temperature with low brake. The supernatant was removed and the pellet was washed again with 40 ml of PBS/EDTA. Then, the pellet was resuspended with 9 ml of PBS/EDTA, was layered

on of 65% of Percoll®/EDTA solution in a proportion of 3:5 and was centrifuged at 400 x *g* for 20 minutes with no brake. After centrifugation, the interphase layer was collected and washed twice with 40 ml of PBS/EDTA at 400 x *g* for 10 minutes at room temperature. Finally, neutrophils were resuspended as mentioned in 2.5.1. section.

2.5.3. Isolation of neutrophils using Lymphoprep™

This method was developed in the present study and it was based on the previous PBMCs isolation protocol explained in 2.2.2. section. Afterwards, exclusively the RBC layer, located underneath the PBMCs and Lymphoprep™ fraction, was collected and diluted with 20 ml of 0.03% sodium bicarbonate for 20 seconds for the lysis of erythrocytes. The isotonicity was restored adding 20ml of PBS/EDTA and neutrophils were pelleted at 300 x *g* for 10 minutes at 4°C. If the presence of erythrocytes observed with the naked eye was suspected, a second lysis step was performed with 5 ml of 0.03% sodium bicarbonate. Finally, neutrophils were washed with PBS/EDTA at 300 x *g* for 10 minutes and resuspended as mentioned in 2.5.1. section.

2.5.4. Morphologic characterization of neutrophils

In order to study the purity of neutrophils in the cell population obtained in each isolation procedure, isolated neutrophils were observed using direct microscopy after Diff-Quick™ staining following manufacturer's instructions (Auto-Hemacolor®). Besides, isolated neutrophils through the three methods already explained, were further characterized through the flow cytometry analysis of 10.000 events (MACSQuant, MiltenyiBiotec®) and the purity was expressed as percentage of neutrophils estimated using SCC:FSC plots, gating the expected location of this population using the MACSQuantify10 Software™.

2.6. Statistical analysis

The total number of isolated PBMCs, the yield of monocytes, number of recovered MDMs with different purification techniques and *in vitro* conditions and the data obtained by

flow cytometry (percentage of purity, percentage of positive cells and fluorescence intensity) were expressed as mean values of the three animals of each animal species and standard deviations, calculated using conventional descriptive statistical procedures.

Multivariate analyses were used according to the nature of the data. Specifically, we used linear models (LM) and generalized linear models (GLMs) with binomial distribution to analyze the effect of the isolation technique (buffy-coat and buffy-coat plus RBC layer) and the animal species (sheep and goats) on the log-transformed number of number of PBMCs isolated and the percentage of purity detected, respectively. At the same time, we used GLMs with binomial and Poisson distributions to explore the effects of cell type (monocyte and MDMs), purification technique (adherence method and immunomagnetic columns), cell phenotype ($CD14^+$, $MCH\text{-}II^+$ and $CD11b^+$) and animal species on the percentage of positive cells and fluorescence intensity detected, respectively. The effect of the animal species and the purification techniques (adherence method and immunomagnetic columns) on the yield of monocytes and the number of recovered MDMs with different purification techniques was assessed using GLMs with binomial and Poisson distributions, respectively. The different variables considered in the yield of MDMs with different *in vitro* conditions, such as cell stimulation with GM-CSF and autologous serum and its different concentrations, and animal species were analyzed with GLMs with binomial distribution. Finally, to analyze the variation observed in the purity of neutrophils (expressed as percentage of neutrophils) estimated in each isolation techniques used (without density gradients, Percoll® and Lymphoprep™) as well as in each animal species, GLMs with binomial distribution were used.

The *dredge, get.models and model.sel* functions included in R package "MuMIn" were used to construct a set of candidate models with all the possible combinations of predictive variables, and according to these, the best model was identified using an automatic selection procedure based on the Akaike Information Criterion corrected for small sample sizes (AICc) (Burnham and Anderson, 2002). The most strong and parsimonious model was selected using the combination of lower AICc and R^2_L (Hosmer-Lemeshow test in binomial regression) and higher R^2 (determination coefficient adjusted by the Kullback-Leibler divergence in Poisson

regression). Subsequently, to evaluate the differences between groups within the relevant variables included in the final fitted model, we used the Tukey's Honesty Significant Difference adjustment for the pair-wise comparisons using the *glht.function* with "multcomp" package in R (Hothorn et al., 2008). *P* values of less than 0.05 were considered statistically significant. All the statistical analyses were performed with the R Software version 3. 3. 2 (R Development Core Team, 2017).

3. Results

3.1. Efficiency of PBMCs isolation

The final cell count and percentage of purity of isolated PBMCs from both isolation techniques (2.2.1. and 2.2.2.) were expressed as mean values and standard deviations by bar plots (Fig. 2A and 2B). The variables animal species and isolation techniques were included in the best ranked model (Supplementary Table 1). Thus, the number of caprine PBMCs isolated from buffy-coat plus RBC layer was significantly greater ($8.50x10^7$ PBMCs on average) than the isolated ones only from buffy-coat ($3.40x10^6$ PBMCs on average) ($\beta = 4.38$, SE = 1.13, $p = 0.018$). However, in sheep, despite the fact that the number of PBMCs isolated from the buffy-coat plus RBC layer was greater ($4.40x10^7$ PBMCs and $1.89x10^7$ PBMCs respectively) no statistical differences were shown ($\beta = 2.87$, SE = 1.11, $p = 0.064$) (Fig. 2A). Furthermore, considering the flow cytometry results (Supplementary Figure 2 and Table 2), when analyzing the isolation technique, the percentage of purity of PBMCs detected from buffy-coat plus RBC layer was also significantly higher compared to buffy-coat alone (90% and 60% on average, respectively) (OR = 1.95, 95%, $p = 0.012$). In turn, despite the fact that no differences in the percentage of purity of PBMCs were observed between both isolation techniques in sheep (92% and 91%), a significant higher purity (96%) of caprine PBMCs was observed when buffy-coat plus RBC was employed in contrast to buffy-coat alone (29%) (Fig. 2B). These pairwise comparisons of the best ranked model can be seen in detail (Supplementary Table 3).

3.2. Monocyte yield according to purification method

The mean values and standard deviations of yield of monocytes were represented (Fig. 3). The purification technique reflected significant differences in the monocytes yield. In fact, the immunomagnetic columns technique showed a significant higher yield percentage of monocytes (18%) than the adherence method (4%) (OR = 4.23, 95%, $p < 0.001$). Additionally, a lower monocytes yield was observed in sheep (1.6% by adherence method and 17% by immunomagnetic columns) in comparison to goats (6 % and 18% respectively) without significant statistical differences (OR = 0.75, 95%, $p = 0.42$) (Supplementary Table 4).

3.3. Efficiency of culture conditions

In the best ranked model, the additive effect of the three variables (animal species, cell stimulation and concentrations) were considered (Supplementary Table 5). No significant differences were observed in the yield of recovered MDMs between animal species (OR = 1.44, 95%, $p = 0.890$). Furthermore, the number of recovered MDMs was higher in those monocytes supplemented with GM-CSF ($5.19x10^5$ MDMs on average) in contrast with those supplemented with autologous serum ($6.21x10^4$ MDMs) (Fig. 4) although no statistical differences were found (OR = 2.71, 95%, $p = 0.839$). Additionally, a gradual increase in the yield was observed as the GM-CSF and autologous serum concentrations were increased (OR = 1.01, 95%, $p = 0.687$).

3.4. Yield MDMs according to the purification techniques

The mean values and standard deviations of the number of recovered MDMs with different purification techniques were represented (Fig. 5). Only the purification technique was included as a variable of interest in the regression models (Supplementary Table 6) and, therefore, the additive effect or interaction with the other variables were not considered. Thus, there was a significant increase in the number of MDMs obtained by the adherence method ($5.47x10^5$ MDMs on average) in comparison to immunomagnetic columns ($3.27x10^5$ MDMs on average) in both animal species ($\beta = 0.21$, SE = 0.06, $p = 0.004$) .

3.5. Phenotypic characterization of monocytes and MDMs

When comparing the size of monocytes purified from sheep and goats on the first day, with the size of MDMs from both animal species after seven days of culture regardless purification technique used, an increase was observed, mainly due to the expansion of the cytoplasm. In addition, two different morphologies were noticed both in sheep and goats: an amoeboid shape or a round aspect. Furthermore, an increase in the granularity could be noticed inside the cytoplasm of MDMs, which was not observed in the monocytes.

An analysis of the morphology and the expression of the surface markers CD14, MHC-II and CD11b were carried out in monocytes and MDMs obtained by adherence method or immunomagnetic columns. Monocyte population showed less SCC and FSC signals than MDMs, in agreement with the increased size and granularity of the latter. Purified monocytes through adherence method appeared more disperse than those monocytes purified through immunomagnetic columns, consistent with the presence of greater cell heterogeneity among those cells purified by adherence method (Fig. 6). However, these differences disappeared when analyzing the MDMs obtained by either technique (Fig. 6).

Descriptive statistics of the percentage of positive cells in relation to the different variables are summarized in Table 1. The animal species and cell phenotype (CD14, MHC-II and CD11b) were included in the worst-ranked models, which showed their little influence on the changes observed in the percentage of positive cells (Supplementary Table 7) and the results were consolidated by the multi-model inference.

Thus, the interaction of the purification techniques with the cell type showed significant effects on the percentage of positive cells detected. Specifically, monocytes purified by immunomagnetic columns showed an increase of the percentage of positive cells (OR = 16.58, 95%, $p = 0.001$) compared to the monocytes purified by the adherence method. However, once maturation of purified monocytes by the adherence method occurs, there was an increase of the percentage of positive cells (OR = 0.41, 95%, $p = 0.003$). All pairwise comparisons of the best ranked model can be seen in the Supplementary Table 8.

Descriptive statistics of the fluorescence intensity in relation to the different variables were summarized in Table 2. The best GLM included cell type, cell phenotype and animal species and its interactions as explanatory variables of interests. The purification technique and its relationship with the rest of the variables were included in the worst-ranked models (Supplementary Table 9). Consequently, the fluorescence intensity of MDMs increased significantly in contrast with the monocytes for the three surface markers evaluated, being MHC-II the surface marker with the greatest fluorescence intensity (Table 2). The differences between different interactions can be seen in supplementary Table 10.

3.6. Isolation of neutrophils

Although neutrophils were present in the samples obtained by the three methods assessed, qualitative differences were revealed when they were observed directly under microscope. A high number of erythrocytes and PBMCs were detected when neutrophils were isolated without density gradients and similar results were observed with the use of Percoll®, including a higher number of PBMCs together with dead cells. However, the isolation of neutrophils through centrifugation with Lymphoprep™ was the method that yielded the higher proportion of neutrophils and the least contamination with PBMCs in both animal species.

Qualitative results mentioned above were supported by purity percentages of the isolated neutrophils estimated by flow cytometry (Fig. 7 and 8). The animal species and the isolation technique influenced the percentage of purity. When both variables were included in the best ranked model (Supplementary Table 11), the Lymphoprep™ technique showed the highest percentage of purity (90% on average) compared to the rest of the techniques used (60% without density gradients and 58% with Percoll®) (OR= 1.72, 95%, p = 0.021). The isolation without density gradients and the isolation with Percoll® did not show significant relevance between them (OR= 0.15, 95%, p = 0.995). In addition, the percentage of neutrophils detected in sheep (46-82%) was significantly lower (OR= 0.45, 95%, p = 0.016) than in goats (66-95%) in the three methods (Fig. 7). The low purity of the neutrophils isolated from sheep isolation without density gradients or with Percoll® was shown by the dispersion of events in the

SCC:FSC plots, where a high number of doublets was observed. In contrast, neutrophils isolated with Lymphoprep™ showed the most homogeneous population in both animal species (Fig. 8).

4. Discussion

The purpose of this work was to compare for the first time different protocols for the isolation of neutrophils and monocytes, and the subsequent generation of MDMs from sheep and goats blood with the aim to propose a standardized and agreed method to be used in these ruminant species. The result of these comparisons is a method that allows the simultaneous isolation of highly pure populations of neutrophils and monocytes from the same sample of blood and the posterior maturation of the latter to MDMs.

Monocytes and macrophages play an important role as the first line of defense against pathogens as well as a link between innate and adaptive immune responses (Auffray et al., 2009). Therefore, their isolation and manipulation under cell culture conditions is an essential tool for the study of the pathogen-host interaction (Altreuther et al., 2001), with great importance in a high number of small ruminant diseases (e.g. paratuberculosis and toxoplasmosis). According to Peterson et al. (2006), the percentage of peripheral blood monocytes in small ruminants is physiologically low (17 ± 24.9% on average). Thus, when a significant number of monocytes is required for *in vitro* studies, the common strategy is to isolate PBMCs and subsequently, purify the monocyte fraction (Marino et al., 2017). The current protocols for PBMCs isolation employed in small ruminants vary widely (Chan et al., 2002; Keane et al., 2002; Peterson et al., 2006; Abendaño et al., 2014); however, among these differences, the centrifugation of diluted blood followed by a centrifugation in a density gradient seems to improve the isolation of PBMCs with a high purity (Keane et al., 2002). Similarly, our results suggest that dilution of blood during the first centrifugation step improves significantly the purity and the number of the PBMCs obtained and, thus, a better further purification of monocytes.

Regarding the purification of monocytes, two different procedures (adherence method and immunomagnetic columns) based on previous studies in ruminant species (Olivier et al.,

2001; Budhia et al., 2006; Machugh et al., 2012; Berg et al., 2013) were here compared in sheep and goats. The adherence method is based on the lack of adhesion capacity of the other cells found in the PBMCs fraction, mainly lymphocytes, which allows their elimination during the washing steps (Taubert et al., 2009; Crespo et al., 2013; Elmowalid et al., 2013). As this procedure is based on the seeding of a high number of PBMCs, the initial population of PBMCs after first washing have a low purity of adherent monocytes. The possible presence of significant amounts of contaminating platelets in our samples of monocytes should be considered, as it has been demonstrated to occur when adherence methods of purification are used (Urata et al., 2008), and this may have an impact on the phenotypic characteristics of monocytes (Beliakova-Bethell et al., 2014; Nielsen et al., 2020). To solve this situation, methods based on a double centrifugation with density gradients have been proposed for removing platelets (Casale and Kaliner., 1982), as well as the performance of consecutive washes, as done in this study, that may also be able to significantly reduce the number of platelets (Ceciliani et al., 2007; Ceciliani et al., 2020). The elimination of monocytes that have not been adhered after three hours of incubation and were removed during the washes might also explain the low yield obtained by adherence method. In contrast, purification of monocytes throughout immunomagnetic columns (Abendaño et al., 2013; Pomeroy et al., 2016) has allowed the collection of an initial monocyte suspension with a high percentage of purity and considerably reduce lymphocyte and platelet contamination (Nielsen et al., 2020). However, the isolation of monocytes using antibodies against CD14 receptor, might entail a lower yield since only a certain type of subpopulations of monocytes would be preselected. Nonetheless, some studies have reported that the $CD14^{+}$ surface marker is highly expressed in all monocyte subsets of sheep and goats (Elnaggar et al., 2016) with the $CD14^{+}CD16^{++}$ subpopulation being the main monocytes subpopulation in sheep (Corripio-Miyar et al., 2015). In addition, it has to be considered that, despite being an antibody raised for human monocyte recognition, a cross-reaction with ruminants monocytes has been previously demonstrated and the use of immunomagnetic columns labelled with CD14 antibody is considered as a useful and effective

tool for the determination of monocytes subpopulations, cytokines profiles and functional studies in ruminants (Budhia et al., 2006; Corripio-Miyar et al., 2015).

Generation of ruminant MDMs takes place under optimal conditions of *in vitro* culture and numerous studies suggest the employment of supplemented medium with autologous serum, cytokines or growth factors in order to facilitate the *in vitro* generation (Keane et al., 2002; Crespo et al., 2013; Wynn et al., 2013). Here in, we have selected the two main procedures previously explained: the use of GM-CSF and autologous serum. The GM-CSF cytokine is expressed mainly in myeloid cell populations, such as monocytes and macrophages favoring their survival, activation and maturation (Rey-Giraud et al., 2012; Draijer et al., 2019) and it has been shown to stimulate the *in vitro* proliferation and maturation of macrophages (Burgess and Metcalf, 1980). Under our experimental conditions, the supplementation with GM-CSF notably improved the generation of MDMs regard to the supplementation with autologous serum. The effect of GM-CSF was more evident in ovine than in caprine MDMs, which may be explained due to the specificity of the ovine GM-CSF used in the stimulation. In this regard, there is not an agreement on the optimal concentration of GM-CSF in the literature (Budhia et al., 2006; Souza, 2015). In our study, increased concentrations of GM-CSF resulted in a higher number of MDMs and we can even assert that GM-CSF was essential for the generation of MDMs. In contrast, other studies showed that GM-CSF was not essential to obtain MDMs and the exclusive use of FBS or autologous serum was enough for the generation of MDMs (Berger and Griffin, 2006; Elmowalid, 2012). Nevertheless, in our study, the use of different autologous serum concentrations led to the obtaining of a lower number of MDMs than the use of GM-CSF.

Remarkably, under the same conditions of maturation (supplementation with GM-CSF), a higher number of MDMs was obtained from purified monocytes with the adherence method than with immunomagnetic columns despite of the purity of monocytes was greater in this latter. This result is clearly in contrast with previous studies, carried out with human PBMCs, where the positive selection of $CD14^{+}$ cells was the preferred method for the generation of MDMs (Nielsen et al., 2020). One possible explanation for the lack of efficiency of magnetic

separation may be related to the low cross-reaction between the human anti-CD14 antibody and the CD14 membrane receptors of ruminants. Nonetheless, this same reagent and the immunomagnetic columns has been employed for the study of MDMs or dendritic cells in sheep and cattle (Budhia et al., 2006; Pomeroy et al., 2016).

In order to analyze the influence of the different purification techniques on the subsequent generation of MDMs, a phenotypical characterization of the cells was carried out. As monocytes and MDMs have a remarkable phenotypical and functional heterogeneity, it is convenient to analyze different parameters, such as morphological changes or the expression of surface markers (Gordon and Taylor, 2005; Crespo et al., 2013) for a further characterization of the cells. In addition, a functional characterization needs to be also carried out. The evaluation of morphological changes was most informative when analyzing MDMs as showed an increase in size and changes in shape, ranging from rounded to amoeboid forms along with an increase in granularity in the cytoplasm. In addition, it seems that purification techniques could have an impact on the morphology of monocytes observed in SCC:FSC plots, since a greater yield of monocytes as well as a more homogeneous population was obtained by immunomagnetic columns than with adherence method. However, the manipulation of purified monocytes during the adhesion and subsequent separation from the plate by mechanical methods might also contributed to alter their morphology and therefore, be associated with a more heterogeneous appearance. Interestingly, no differences were observed in MDMs morphology coming from monocytes purified by adherence method or immunomagnetic columns. This analysis proved the usefulness of the morphological characterization of MDMs obtained under *in vitro* conditions (Olivier et al., 2001; Weiss et al., 2002; Jensen et al., 2014). In addition, the analysis of surface markers gave us information about purity of both cell populations (monocytes and MDMs). The analysis of CD14, MHC-II and CD11b showed that purification of monocytes through immunomagnetic columns produced a higher percentage of cells expressing these markers than those isolated by adherence method. Immunomagnetic enrichment has been proven to be an efficient procedure to select $CD14^+$ cells, allowing the obtaining, in sheep and cattle, cell populations with a 80-90% of $CD14^+$ cells (Budhia et al., 2006; Jensen et al., 2014)

from a PBMC fraction that usually contains 4.1% of $CD14^+$ cells (Corripio-Miyar et al., 2015). However, it should be also noted that small ruminant granulocytes could also express CD14 (Elnaggar et al., 2016), which could reduce the specificity of the immunomagnetic purification of monocytes. Thus, it is important to analyze other surface markers that could help to evaluate the phenotypic characterization of cells and purity (Chan et al., 2002; Elmowalid, 2012). The adherence method led a lower percentage of positive cells expressing CD14, MHC-II and CD11b surface markers. Rather than purifying less number of cells expressing these markers, it is probable that the low percentage of positive cells obtained was a consequence of the higher number of cells that remained adhered to the surface of the plate during the first hours after seeding and that would be lost in the subsequent washing steps. Similarly, activation of monocytes caused with the adherence procedure or the influence of the higher proportion of contaminating lymphocytes, could also alter the expression of surface markers and/or cytokines (Nielsen et al., 2020) which could also explain differences here observed.

These findings are in contrast with the results obtained in previous studies where monocytes purified by adherence method gave a purity of 93.5% (Elmowalid, 2012). However, there are significant methodological differences such as the technique used to assess phenotypic characterization (indirect fluorescence assay under fluorescent microscopy), which may explain these discrepancies. Most authors carry out CD14 analysis in order to characterize monocytes purified both by immunomagnetic columns and adherence method (Olivier et al., 2001; Budhia et al., 2006; García-Sanchez et al., 2019) but the preselection of $CD14^+$ monocytes could justify the high percentage of positive cells, consequently, this study also analyzes the expression of MHC-II and CD11b to supports that $CD14^+$ cells isolated were consistent with monocytes. However, in the study carried out by Nielsen et al. (2020), it was observed a decrease in the expression of CD14 after the use of immunomagnetic columns which was associated with the internalization of the receptors due to the binding with the CD14 conjugated microbeads. Moreover, it is known that as adherence method, the bounding between the CD14 antibody coated into the magnetic beads and the CD14 membrane molecule may change the phenotype of

monocytes, either in their morphology or in the cytokines that they produce, such as IFN-γ, TNF-α or IL-12 (Fikri et al., 2000; Kowalewicz-Kulbat et al., 2016).

Regarding the analysis of surface markers in MDMs, there was a clear difference in the measurement of the fluorescence intensity labelling of all surface markers between monocytes and MDMs, regardless the purification technique. This result is in agreement with Olivier et al. (2001), who described an increase in the intensity of expression of CD14 and MHC-II in MDMs after four days of cell culture, in contrast to monocytes. Furthermore, the increase in expression of MHC-II and CD11b has been described in numerous studies as a reliable indicator of monocyte maturation (Berger and Griffin, 2006; Elmowalid, 2012).

There are several protocols published in the literature for the isolation of neutrophils with a wide variation on their methodologies (Paltrinieri et al., 2000; Siemsen et al., 2014). Among three protocols here employed, the isolation of neutrophils without density gradients was ineffective (50-60% of purity) due to the great contamination with erythrocytes and PBMCs in both species. Although the gate established for neutrophil identification at flow cytometry, by size and granularity, suggested a 50-60% of purity among isolated neutrophils from both goats and sheep, a subsequent visual analysis through Diff Quick™ staining showed a high contamination. Neutrophils isolated from sheep showed large numbers of erythrocytes and dead lymphocytes, while in goats, the number of neutrophils was higher but they were highly contaminated with PBMCs. Therefore, purity obtained by this method was very low, which disagrees with the purity above 60-70% in sheep using similar protocols (Mulder and Colditz, 1993; Sartorelli et al., 1999; Paltrinieri et al., 2000), which could be explained by differences in the methodology (lysis buffer and/or evaluation of further purity). Nevertheless, a purity around 60% was obtained when processing bovine blood with similar methods (Ordóñez et al., 2008). In this sense, previous studies claimed that the main contamination of isolated neutrophils was due to lymphocytes, as they could make up to 30% of the cells from the RBC fraction, so centrifugation with Percoll® as a density gradient was advised in order to avoid contamination by PBMCs (Woldehiwet et al., 2003). The use of Percoll® in the current study improved the purity of neutrophils in both species despite of a significant contamination by erythrocytes and

cellular debris, which was more marked in sheep and might be explained by the different density of ovine and caprine neutrophils (Siemsen et al., 2014). Despite previous results showed a high efficiency, the use of Percoll® at 65% (Woldehiwet et al., 2003; Siemsen et al., 2014) did not allowed us the isolation of a pure population of neutrophils. The reasons for this discrepancy are not clear, although small differences in the methods applied might be involved.

The third protocol here employed for neutrophils isolation using Lymphoprep™ achieved an effective method for the isolation of small ruminant PBMCs and further purification of monocytes that can also permit the separation of RBCs fraction and PBMCs. The results showed a purity of 80% in sheep and 90% in goats and with a minimal contamination of PBMCs. Silva et al. (2014) proposed a similar protocol for the isolation of caprine neutrophils using Biocoll® instead of Lymphoprep™, although the purity was not estimated. These results are in contrast with those obtained by Woldehiwet et al. (2003), who using Percoll® at 55% (with an identical density to Lymphoprep™ or Biocoll®) achieved a lower purity of neutrophils than using concentrations of Percoll® above 65%.

5. Conclusions

The proposed protocol based on the use of Lymphoprep™ allows the simultaneous isolation of PBMCs and neutrophils in sheep and goats with a high purity. The PBMCs obtained could be further purified for the purification of monocytes, and their subsequent generation of MDMs by both adherence method and immunomagnetic columns. Although the purification with immunomagnetic columns leads a population with a higher purity of monocytes than the adherence method, this latter allows the obtaining of a greater number of MDMs. Thus, a reliable and efficient protocol for the isolation of neutrophils and monocytes has been proposed in this work together with the establishment of the optimal conditions for the subsequent *in vitro* generation of MDMs, with the aim of standardize the procedures to be used when the study of these cell populations is required in small ruminants.

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Figure captions

Fig. 1. Scheme of protocols of PBMC isolation techniques from buffy-coat alone (2.2.1. section) and buffy-coat plus RBC (2.2.2. section). Each protocol was carried out using 200 ml of blood. Isolated PBMCs were washed three times before counting and flow cytometry analysis.

Fig. 2. Efficiency of PBMCs isolation by two different methods in sheep and goats from the same blood sample (200 ml). (A) Total number of isolated PBMCs. (B) Percentage of purity of isolated PBMCs. Bars represent the mean values whereas the vertical lines represent the standard deviations. * and ** symbols indicate $p < 0.05$ and $p < 0.01$ significant differences, respectively.

Fig. 3. Yield of monocytes purified by adherence method (AM) and immunomagnetic columns (IC) in sheep and goats. Data are expressed as mean values and vertical lines represent standard deviations. *** Symbol represents a significant difference of $p < 0.001$.

Fig. 4. Yield of MDMs in sheep and goats obtained from purified monocytes by adherence method with different cell culture stimulations and concentrations. Bars represent mean values of total number of MDMs and vertical lines the standard deviations. No significant differences were shown between GM-CSF and autologous serum (AUS) neither between sheep and goats.

Fig. 5. Yield of MDMs obtained from monocytes purified by adherence method (AM) and immunomagnetic columns (IC) in sheep and goats and supplemented with GM-CSF (60 ng/µl). Bars and vertical lines represent mean values and standard deviations respectively of the total number of recovered MDMs. Significant difference is expressed as ** symbol ($p < 0.01$).

Fig. 6. Analysis of size (SCC) and complexity (FSC) of monocytes and MDMs in sheep and goats carried out by flow cytometry. Purified monocytes from sheep and goats by both immunomagnetic columns (IC) and adherence method (AM), together with ovine and caprine MDMs from purified monocytes by IC and AM, respectively and supplemented with GM-CSF (60 ng/ml) are represented in dot plots. A greater heterogeneity is observed in monocytes purified by means of the AM in both species in contrast with the well-defined population achieved through IC delimited by the red gate. Percentage of events (SSC:FSC) and mean percentage of $CD14^+$, $MHC\text{-}II^+$ and $CD11b^+$ are summarized in each plot. No differences between purification techniques of monocytes neither animal species were observed in MDMs populations.

Fig. 7. Efficiency of neutrophils isolation technique (IT) expressed as percentage of purity estimated by flow cytometry. Bars represent mean values of percentage of neutrophils isolated without density gradients (WDG), using Percoll® and using Lymphoprep™. Vertical lines represent standard deviations. * Symbol indicates a significant difference of $p < 0.05$.

Fig. 8. Analysis of size (SCC) and complexity (FSC) of isolated neutrophils by three different protocols carried out in sheep and goats by flow cytometry. Neutrophils were isolated without density gradients (WDG), using Percoll® and using Lymphoprep™. Neutrophils are represented inside the red gate. Notice the high degree of heterogeneity of cells isolated WDG and using Percoll®, which it was more marked in sheep than in goats and did not correspond to the neutrophils gate. Lymphoprep™ achieved a well-defined and abundant population of cells corresponding to neutrophils observed inside the gate in both species.

Figure 1

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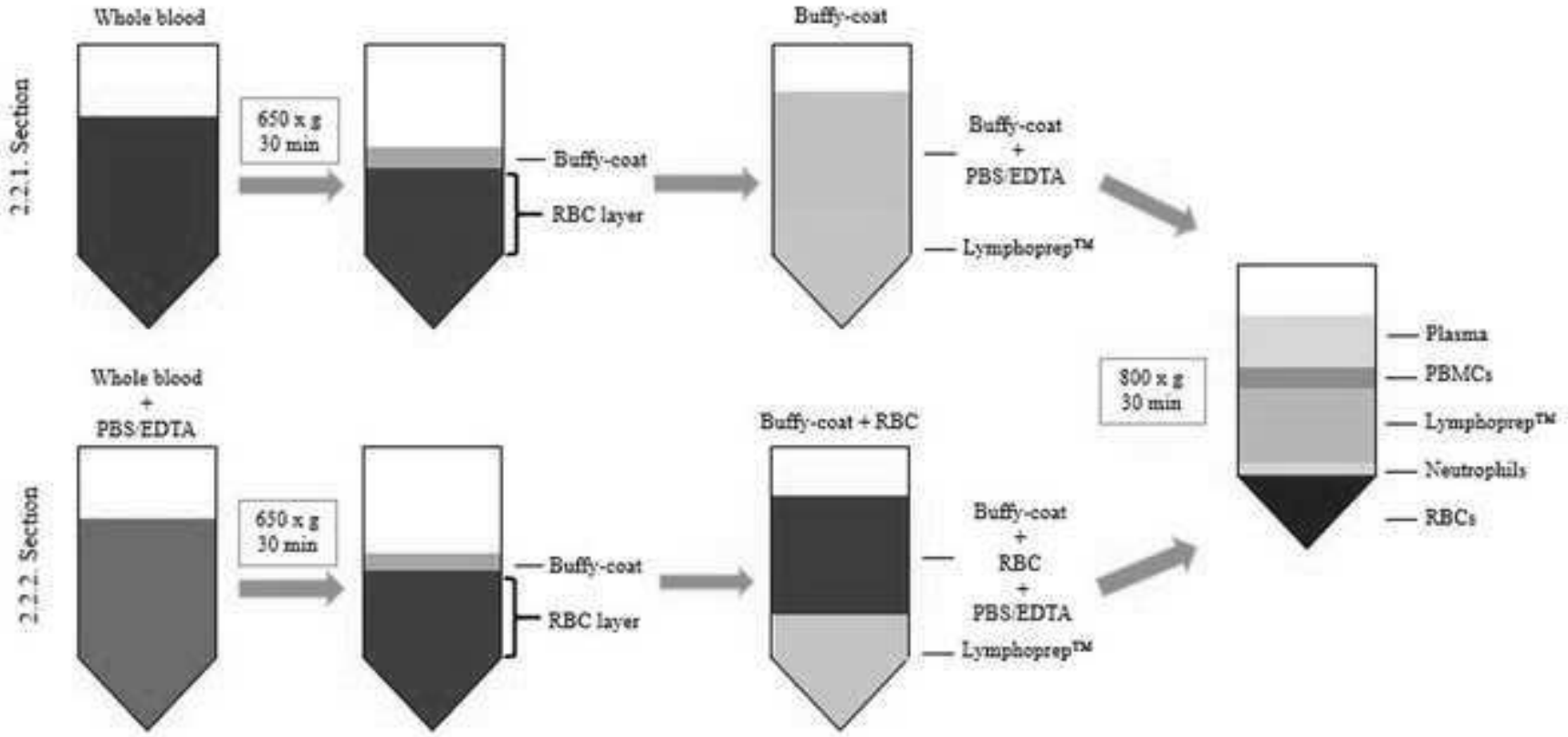

Figure 2

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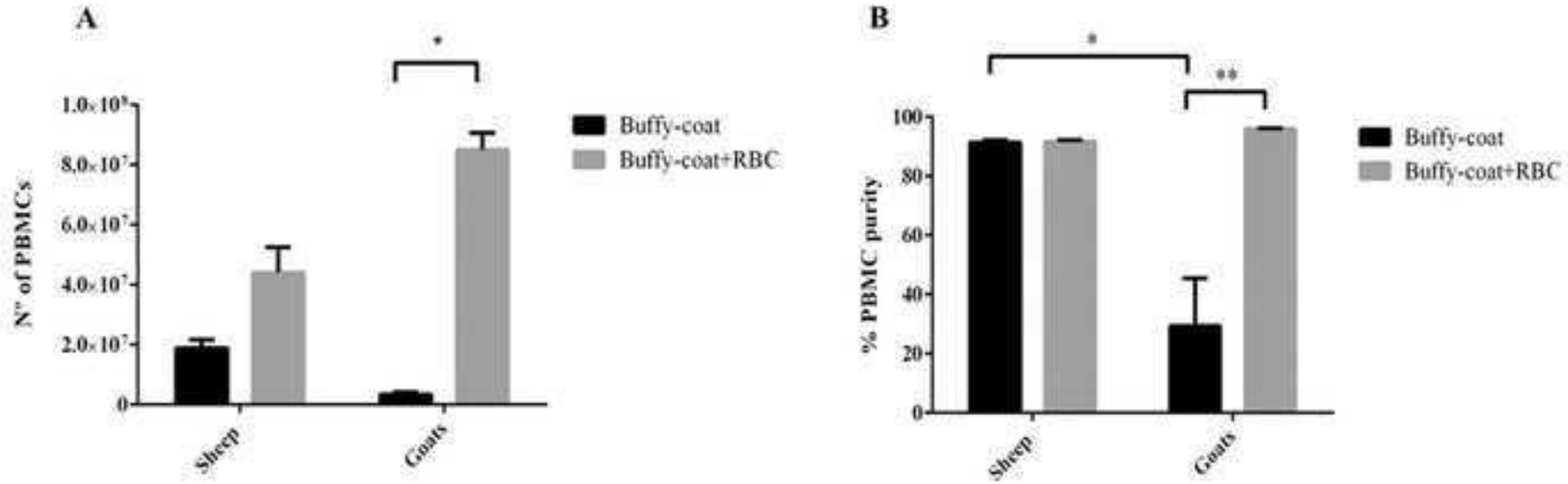

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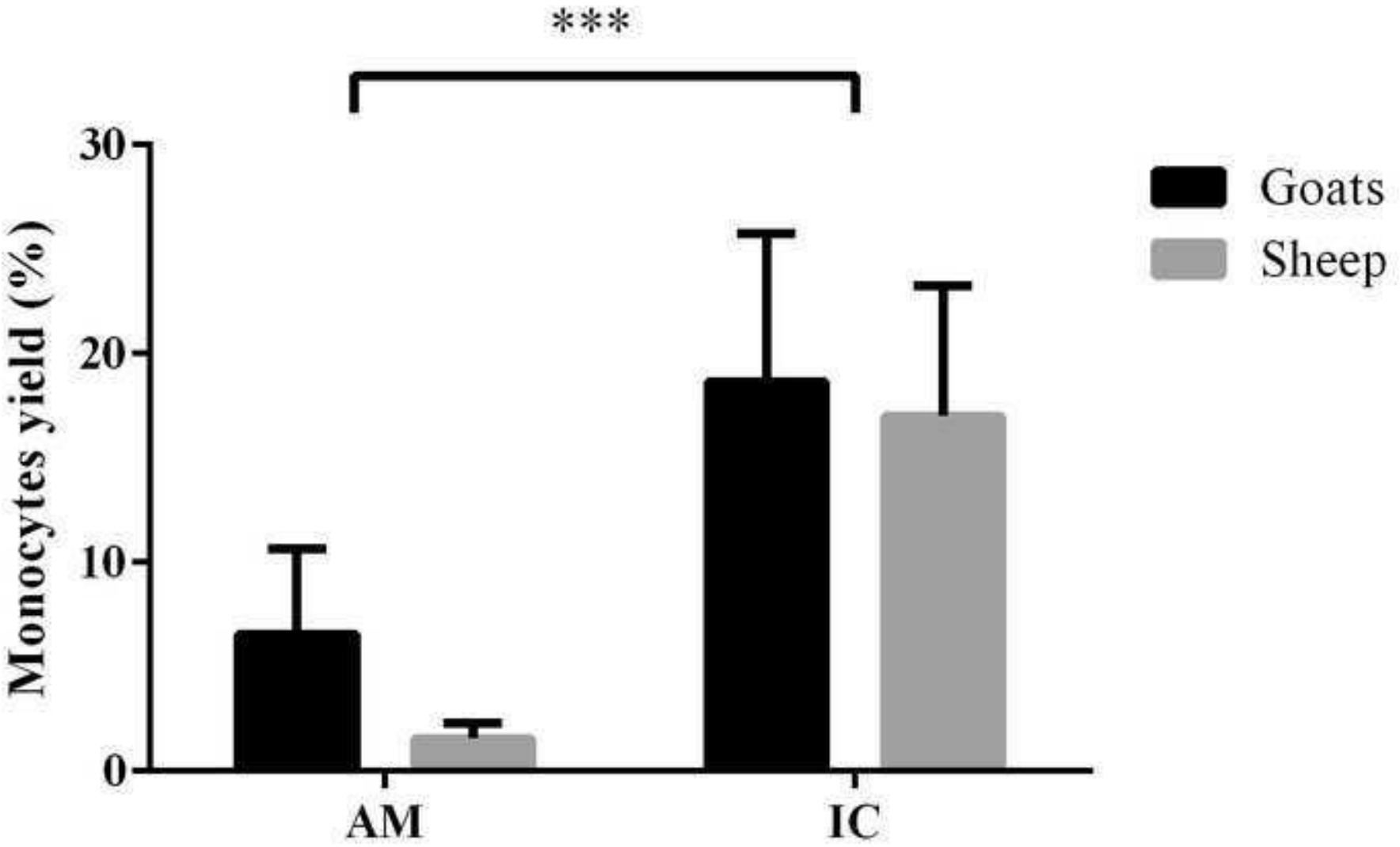

Figure 4

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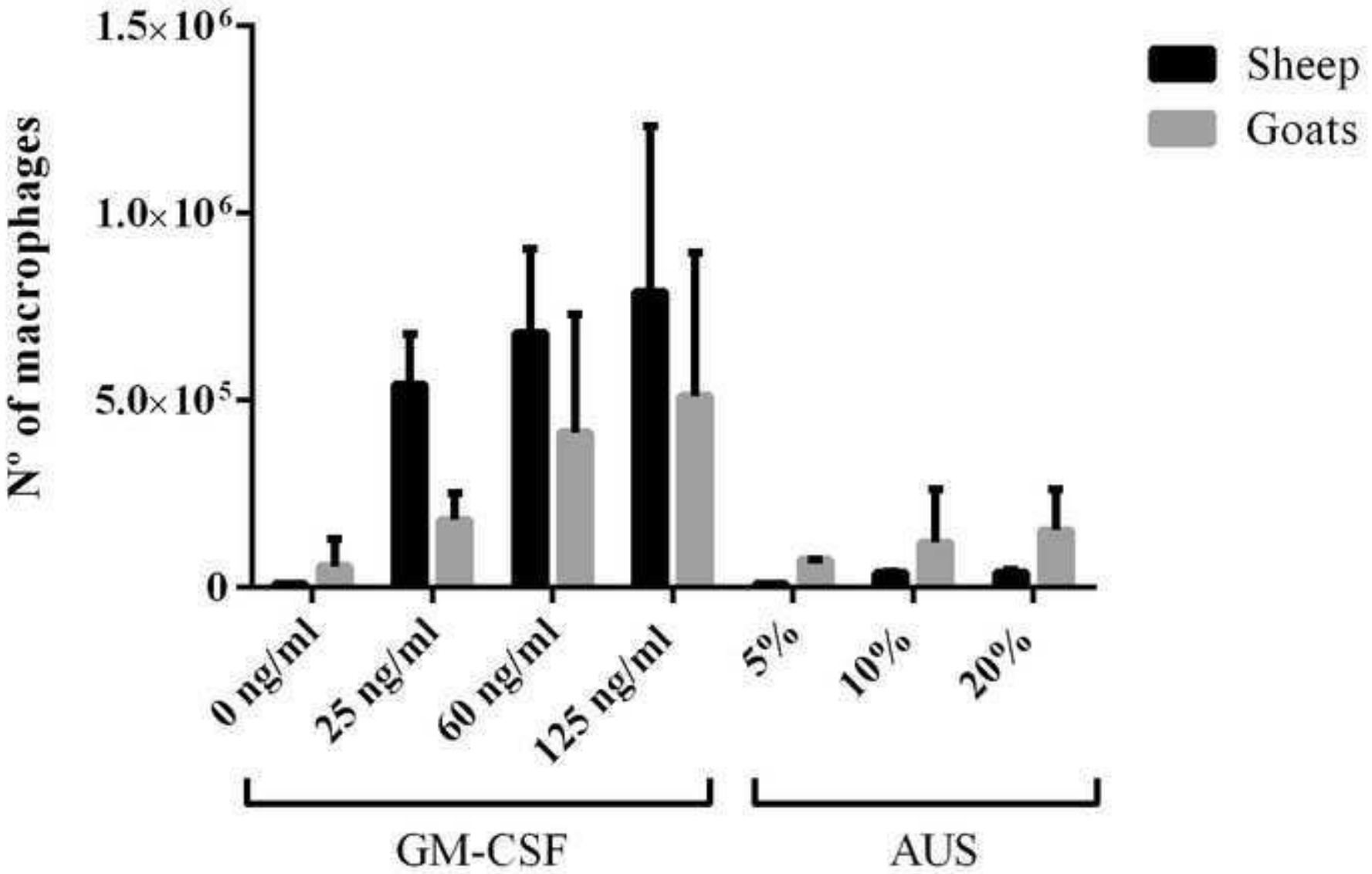

Figure 5

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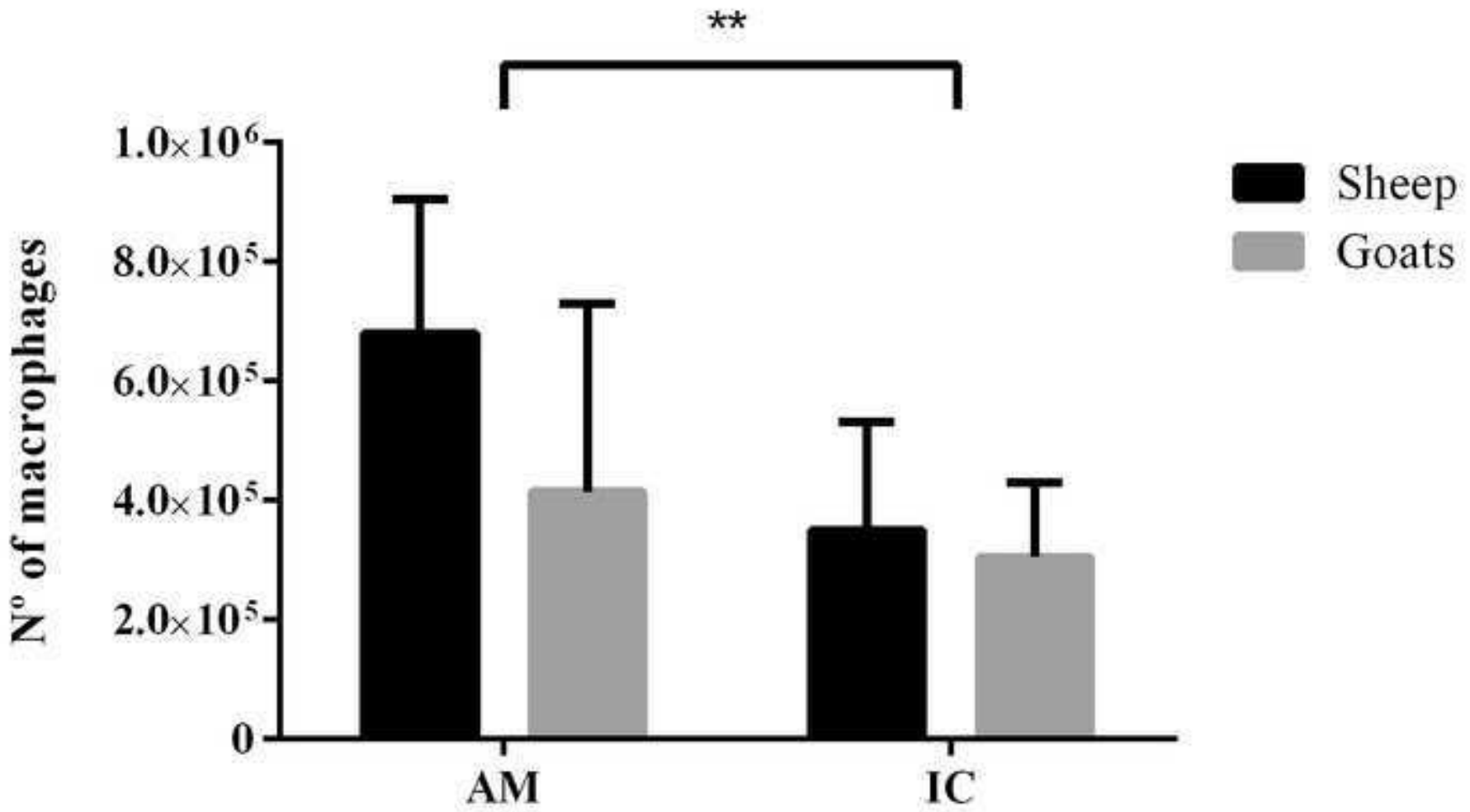

Figure 6

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