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A Simple Method for Generation of Functional Sheep Peripheral Blood Monocytes-Derived-Macrophages *In vitro*

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Abstract: Macrophages are professional antigen presenting cells cooperating with other immune cells for the activation of innate and adaptive immune responses. The objective of this study was to establish simple functional sheep macrophages culture system. Peripheral blood monocytes were cultured for 3-4 days on gelatin/autologous plasma coated surfaces in slightly acidic media supplemented with 5-10% autologous serum. The cells morphological, phenotypical and functional characteristics were investigated. Over 3-4 days in culture, the monocytes differentiated into monocytes-derived-macrophages (MDM). These MDM increased 3-4x in size compared to monocytes, contained many cytoplasmic granules and had kidney shape nuclei. Significant increase in the expression level of MHC II, CD11b and CD11c surface markers was observed, meanwhile, MHC I and CD14 expression levels were similar. MDM had 2-5x more phagocytic capacity and had 2-3x microbicidal activity (fungicidal and bactericidal) compared to the monocytes. MDM were also able to produce oxygen radicals and the level of production was insignificantly different in both monocytes and MDM. In conclusion, the method described here is economic and convenient to produce large number of functional sheep MDM in short period of time. This culture system could be used to study the host-pathogen interaction and could be used in many immunological studies.

Key words: TRITC Labeled Yeast · Nitric Oxide Production · Phagocytosis And Microbicidal Activity

INTRODUCTION

Differentiated tissue macrophages arise from monocytes recruited from the blood [1]. Once differentiated, macrophages become long-lived cells and develop specialized functions. Macrophages mediate innate immune responses and contribute to adaptive immunity via antigen processing and play a pivotal role in a number of important pathophysiological processes. These processes include tissue repair, antigen presentation, lymphocyte activation and host defense against infectious diseases and directing local and systemic immune responses [2]. These biological functions require the successful completion of the differentiation pathway from immature precursor to mature macrophage [3]. Once differentiated, macrophages become long-lived cells and develop specialized functions [2, 3]. However, tissue macrophages are essentially unobtainable and if obtained, it would be in few numbers and would hardly to be maintained *in vitro*.

Generation of macrophages from blood monocytes *in vitro* considered a good tool for studying the interaction of pathogens with macrophages and was taken as a model to mimic tissue macrophages. This *in vitro* maturation of macrophages has also proven to be a very useful model in both laboratory animals and humans for studying host cell-pathogens interaction [4-6]. The most commonly used methods for separating monocytes are based upon either physical (density) or functional (adherence) properties of these cells. Methods based on cell density are often not used due to high costs [7]. These methods are frequently time consuming and often the enrichment and yield of human monocytes are poor [8]. Poor yield was also a problem when density was used for bovine monocytes separation [9]. With using the adherence methods for separation of human, laboratory animals or bovine peripheral blood monocytes, the major problem is recovery of intact cells because physical methods (scraping) have to be used and this can adversely affect viability and yield [5, 10].

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Macrophages exhibit marked phenotypic heterogeneity [11] and functional diversity, which result from the differentiation programs of these cells and the surrounding micro environmental conditions [2]. Major functions of macrophages include maintaining tissue homeostasis, responding to micro-organisms and are able to produce oxygen metabolites [1]. Macrophages or monocytes activation in different animal species has been shown to produce cytokines. Among these Cytokines are interferon (IFN)-γ and tumor necrosis factor (TNF)-α, (IL)-4, IL-10, and IL-13, which lead to, regulation of the immune response, enhancement of phagocytic behavior and anti-inflammatory roles [12-14].

Previous studies have used interferon-γ, interleuin-2 and interleuin-4 as growth factors for monocytes cell differentiation to macrophages and/or to dendritic cells, but these methods were costly with poor yield and affect cells viability. In this study, a simple reproducible culture system that allows monocytes to mature and to differentiate into macrophages was described. Analysis of cell morphology, expression of surface markers; phagocytic capacity; oxygen metabolites; and microbicidal activity confirmed that the differentiated cells resembled the tissues MDM. Using the standardized culture conditions, normal sheep macrophages can be used as an in *vitro* model to study cell-pathogen interaction, immunomodulators interaction with macrophages and to study drug and vaccine delivery by macrophages.

MATERIALS AND METHODS

Animals: Sixty healthy young and adult (6-24 month old), Balady sheep of either sex were used in this study. Sheep were housed at Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia Governerate, Egypt. Sheep were used for peripheral blood mononuclear cells, serum, or plasma collection.

Experimental Design: Blood was collected one day prior to peripheral blood mononuclear cells separation for both autologous serum and plasma collection. The harvested sera and/or plasma were kept at 4°C and used either in the second day or within one week at maximum. Directly in the second day or within one week, blood was collected from the same animals and mononuclear cells were separated. For the sake of reproducibility, blood was collected from at least five sheep in each experiment.

For monocytes deriving to macrophages, many micro-environmental cultural conditions had been tested in monocytes cell culture. Here, only the optimal conditions that resulted in full deriving of monocytes to functional macrophages will be described. To follow up the monocytes development and maturation to MDM, the cells were examined daily for any morphological, phenotypical and functional changes, but the changes at day one and four of culture will be discussed. Although the cells were mature by the third to fourth day of culture, the results of cell development at the 1st and 4th days will be detected. Monocytes yield and detachment were also tested and results are explained in the discussion section.

Autologous Plasma and Serum: Heparinized blood was collected by jugular venipuncture using a 20 cc syringe containing heparin sulfate (Sigma, Sigma Chemicals, St. Louis, MO) for autologous plasma separation or without heparin for autologous serum separation (AS). The non heparinized blood was allowed to clot for 2 h at 37°C followed by 30 min at 4°C. Subsequently, the heparinized or non heparinized blood was centrifuged at 800xg for 30 min at 4°C. After centrifugation, the plasma or serum was collected and centrifuged at 500xg for 20 min at 4°C. plasma or serum was filter-sterilized through 0.45 µm filter (Millipore). The plasma or serum for each animal was labeled and stored separately at 4°C overnight or for a maximum one week. Serum was heat-inactivated before storage.

Separation of Peripheral Blood Monocytes: Sheep peripheral blood monocytes were isolated according to the method described by [9] with many modifications to get good yield. In brief, 20 ml of heparinized sheep venous peripheral blood was diluted and overlaid on histopaque 1083 (Sigma, St. Louis, MO, U.S.A) in a 50-ml tube and centrifuged at 800 xg for 30 min at room temperature. The PBMCs were aspirated from the inter-phase layer, diluted with heparinized PBS and then pelleted by centrifugation at 600 xg for 15 min at 4°C. After being washed in heparinized PBS, the cells resuspended in medium 199 (Life Technologies, Grand Island, NY, U.S.A) containing 10 % heat-inactivated autologous serum (AS), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin-B (25 µg/ml). Cell viability was assessed by trypan blue (Lonza, Allendale, NJ, U.S.A) and it was > 90%.

After being counted, the cell was plated at density of 4-5x10^6 cells/ml into gelatin/plasma-coated [(previously coated with 500 µl/well of 2% gelatin type B, Sigma, St Louis, MO, for 3 h followed by 300 µl/well autologous plasma for 1 h) -24-well plate. The plate was incubated for 2 at 37°C in a humidified CO_2 incubator with gentle rocking every 30 min. After incubation, the media containing non-adherent cells were removed by aspiration and the plate was washed with 37°C pre-warmed PBS to get adherent monocytes.

Generation of Monocytes-Derived Macrophages: Generation of functional monocytes-derived macrophages (MDM) was done by using adherent monocytes that separated as described by [9]. Briefly, 1 ml/well of complete, slightly acidic (pH 6.7), RPMI-1640 (Life Technologies, Grand Island, NY), containing 10 % autologous serum and penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (25 µg/ml) was added to the adherent monocytes and the cells were incubated for 24 h at 37°C in a humidified incubator under 5 % CO_2 tension. After incubation, the conditioned culture media were removed and replenished by 500 µl/well of fresh pre-warmed complete RPMI-1640 (pH 6.7); 5 % AS and antimicrobials. The conditioned media were centrifuged at 800 xg for 20 min at room temperature to pellet the non-adherent cells and any cell debris. Then, 500 µl of conditioned media was added to each well for a total of 1 ml/well. The adherent cells were cultured for an additional 48-72 h to allow monocytes to mature to functional macrophages with the media being changed every 24 h as described. The cells were examined every other day for morphological, phenotypic and functional changes.

Characterization of MDM

Morphological Characterization: The cells were stained using Quick Wright Giemsa stain (Sigma, St. Louis, MO) according to the manufacturer's instructions at the 1st and 4th day of culture in the 24-well plate or in chamber glass slides. Stained cells morphological changes were examined under light microscope or directly without staining under inverted microscope.

Phenotypic Characterization by Indirect Fluorescence Assay: Indirect fluorescence assay (IFA) staining for MHC I, MHC II, CD11b, CD11c, or CD14 expression was done in 24-well plates according to the method described by [15, 16]. The cells were washed with washing buffer of PBS containing: 0.2 % bovine serum albumin (BSA, Sigma, St. Louis, MO) and 0.1 % sodium azide (Aldrich Chemical Company, Milwaukee, WI). Then, the cells were fixed by adding 200 µl of freshly prepared 2 % cold paraformaldehyde (PFD, Aldrich Chemical Company, Milwaukee, WI) to each well, then, the cells were incubated for 10 min at room temperature. Cells were washed in washing buffer and pre-incubated with staining buffer containing: 10 % fetal calf serum (FCS, Highclone, Logan, UT) for 20 min at room temperature. The cells were then incubated with saturating concentrations of each mAbs [(1:600 for MHC-I and 1:200 for MHC-II, CD11b, CD11c, or CD14 (VMRD, Pullman, WA)] for 20 min at 4°C. After being washed, cells were incubated with 1:500 FITC-conjugated Fab fragment goat-anti-mouse IgG (ICN/Cappel Laboratories, Costa Mesa, CA) for 20 min at 4°C. The cells were washed and fixed with 200 µl/well freshly prepared 2 % PFD. Green fluorescent cells (positive) were counted in 15 random microscopic fields of total 300-350 cells and percentage of positive cells for each surface marker was calculated.

Functional Characterization

Cells Phagocytic Activity: The phagocytic activity of MDM was assessed in 24-well gelatin/plasma coated plates. TRITC-labeled-*C.albicans* was prepared according tothe method of [16] and was used for measuring MDM phagocytic activity. Monocytes or MDM containing TRITC-labeled-yeast were observed under fluorescent microscopy and photomicrographs were taken. Monocytes or MDM in 15 random fields containing total cell number 300-350 cells were counted and the number of yeast/cell was counted. The phagocytosis percentage was calculated according to the following equation:

$$\text{Phagocytosis \%} = \frac{\text{Number of MDM containing any No. of yeast}}{\text{Total number of monocytes or MDM cells}} \text{X } 100$$

To determine the phagocytic capacity, monocytes or MDM cells containing ≥ 10, ≥ 25, or ≥ 40 FITC-labeled yeast/cell in at least 20 random microscopic fields (average 200-250 monocytes or MDM) were counted and their percentage was calculated.

Oxidative Burst Assay: The MDM oxidative burst activity was measured in 24-well plate using propidium iodide (PI) labeled-*Escherichia coli* at the 3rd or 4th day of culture. *Escherichia coli* (*E. coli*, strain O55, ATTC, Rockville, MD) were plated, propagated and counted, then labeled with PI according the method of [17].

Cells were examined under fluorescent microscopy and then counted in 15 randomly selected microscopic fields and photomicrographs were taken. The percentage of oxidative burst positive cells was calculated according to the following equation:

$$\text{Percentage of oxidative burst} = \frac{\text{Number of positive monocytes or MDM}}{\text{Total number of counted monocytes or MDM}}$$

Nitric Oxide Assay: Nitric oxide (NO) production by monocytes in the 1st day or MDM in the 4th day was done in 24-well plate. Monocytes or MDM were stimulated with 5 µg/ml of *E. coli* 0111:B4 lipopolysaccharide (LPS, Sigma, St. Louis, MO) in nitrite or nitrate free RPMI 1640 as confirmed with Griess reagent (Sigma, St. Louis, MO). The cells were incubated for 12, 24, or 48 h at 37°C under 5 % CO_2. At the predetermined time, NO produced in the culture media was determined by assaying its stable end product, NO_2 (nitrite) according to the method of [18] using Griess reagent. A standard curve was obtained by use of twofold serial dilutions (1.95-1000 µmols) of sodium nitrite ($Na\ NO_2$) concentrations. The nitrite concentrations were determined by measuring the optical density at 540 nm and comparing the optical density values with values on $Na\ NO_2$ standard curve. The amount of NO produced by monocytes or MDM was estimated in µmols of nitrite.

Intracellular Survival Assay: An intracellular survival assay was performed to measure monocytes or MDM microbicidal (fungicidal or bactericidal) activity. The cells ability to kill yeast or bacteria was done based on the method of [19] with many modifications.

Fungicidal Activity: Monocytes or MDM fungicidal activity was assessed using intracellular survival assay. The cells were cultured in 24-well plate; washed 2x with RPMI-1640 containing no fungicidal agents; and then 300 µl of fresh RPMI-1640 containing 10 % autologous sera and *C. albicans* at ratio of 20 yeast/cell was added per well. After incubation for 30 min at 37°C, the cells were washed 3x with cold PBS to stop phagocytosis and to remove the extracellular yeast. After removing extracellular yeast, 100 µl of sterile 0.5% saponin were added to half of the inoculated wells (total of six wells) to lyse the monocytes or MDM and 1 ml of RPMI 1640, containing 10% AS was added to each well of the remaining three wells and the plates were incubated at 37°C for 10 min. The lysed monocytes or MDM were harvested by aspiration and added to 200 µl of 2x YM broths and stored at 4°C and considered as (time 0). The remaining three wells were further incubated for 90 min for a total of 120 min, subsequently, the medium was removed and 100 µl of sterile 0.5% saponin and cell lysed as mentioned and this consider time 2h. The lysed cell suspension at time (0) or at time 2h was added to 100 µl of 2xYM broth in 96-well plate and the plate was incubated at 37°C for 12 h. After incubation, 30 µl (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO) of 50 mg/ml, was added to each well and the plate incubated at 37°C for 2 h. Optical density was measured at 540 nm wavelengths in ELISA reader. The percentage of cell killing was calculated by the following equation:

$$\text{Percentage of survival} = \frac{[1 - \text{absorbance after incubation for 2 h}]}{\text{Absorbance at time 0}} \times 100$$

Bactericidal Activity: Bactericidal activity against *E. coli* was evaluated for monocytes or MDM as described above for fungicidal activity but LB broth was used rather than YM broth and the bacteria were cultured for 18 hr at 37°C rather than 12 h. The MTT was added and incubated with the bacteria for at 37°C for 6 h rather than 2 h.

Statistical Analysis: Data was analyzed by student's t-test to assess the significance of the differences between mean values of obtained on day 1 and day 4 of culture. Differences were considered significant at $P < 0.05$.

RESULTS

Cells Morphological Development: Monocytes or MDM morphological changes are shown in Photo. 1 A and D. On the 1st day, the monocytes were rounded or ovoid in shape with few dendritic projections and membrane roughening (A and C); their nuclei were large and dense and the cytoplasm: nuclei ratio was low and their sizes were 6-8 µm (C). While on the 4th day of culture, the cells reached a maximum state of differentiation and growth and they morphologically developed into macrophages. MDM were round in shape with increase in membrane roughness and had amoeboid shape; had relative increase in the cytoplasmic: nuclear ratio; had large U-shaped nuclei with pre-nuclear fine granules appeared and their sizes increased to 18-23 µm (B and D). This experiment was repeated for at least five times and the cells were examined directly and/or stained with similar morphological characters each time of examination.

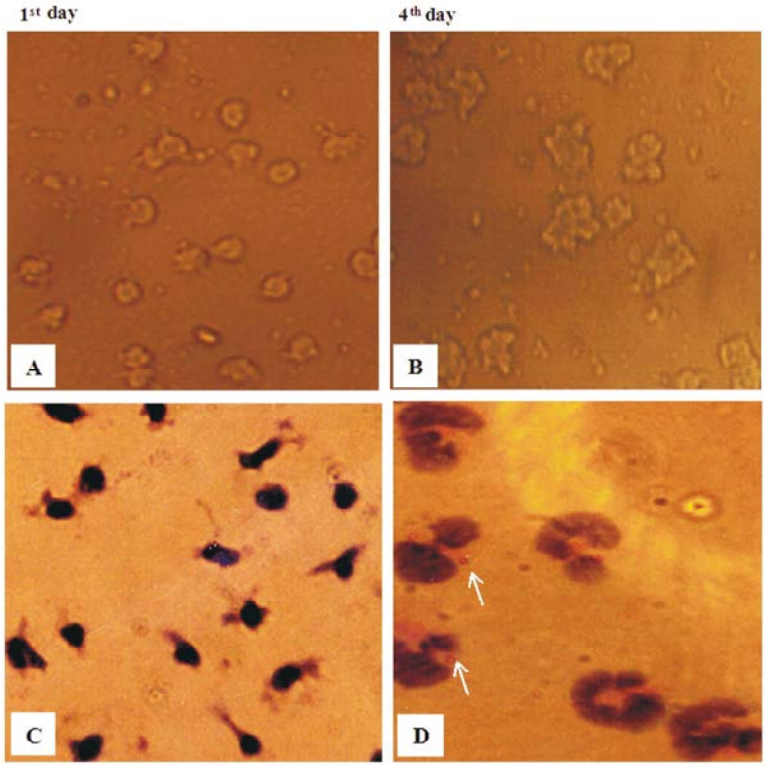


Photo 1: Monocytes-derived macrophages morphological changes. Cells were examined directly under inverted microscope (A and B) or stained using Giemsa's stain at the 1st and 4th day of culture and examined light microscope (C and D), at total magnification of 400x and 900x. At the 1st day cells were small in size and narrow cytoplasmic space and few dendrites (A and C), while at the 4th day, they had membrane roughening and bigger sizes (B and D) with cytoplasmic granules (white arrows, D) and the cytoplasm: nucleus area was wide (D).

Table 1: Cell surface markers expression in both monocytes (1st day) and MDM (4th day). Day of culture

Surface Marker	1st day	4th day
MHC I	97.2±1.0%	98.2±1.3%
MHC II	69.7±7.5%	96.6±4.7%
CD11b	34.5±3.9%	*93.7±2.5%
CD11c	33.7±5.5%	*94.8± 2.5%
CD14	93.5±6.5%	98.5±1.1%

Blood was collected from at five sheep and cell surface markers were analyzed for each individual animal

* The results were significant at $P < 0.05$

Cell Surface Markers Development: Expression levels of the cell surface markers at the 1st and 4th day of culture are shown in Table 1. The percentage of MHC I expression was 97.2±1.0 % and 98.2±1.3 % in monocytes and MDM, respectively. The level of MHC II expression was 69.7±7.5 % on the 1st day of culture, while on the 4th day, MHC II expression level increased to 96.6±4.7 %. The level of CD11b expression increased from 34.5±3.9 % on the 1st day 93.7±2.5 % on the 4th day. Meanwhile, CD11c expression level was 33.7±5.5 % on the 1st day and increased to 94.8±2.5 % on 4th day of culture. CD14 expression level was similar in both cell types and it was higher than 90% at the 1st, or 4th day of culture (Table 1). The fluorescent intensity of surface markers on the 1st or 4th day is shown on Photo 2.

Cells Phagocytic Activity: Phagocytic activity was measured at the 1st (monocytes) and 4th day (MDM) of culture using TRITC labeled yeast. On the 1st day, over 98 % of monocytes were actively phagocytize the yeast. A dramatic increase in the MDM (4th day) phagocytic capacity was found compared to monocytes (1st day). On the 1st day, there was no significant difference at $p < 0.05$ between monocytes and MDM, where the phagocytic capacity of cells contained ≥ 10 labeled yeast/cell was 92.8±4.0 % and 98.0±1.5%, respectively (Table 2). However, there was significant difference at

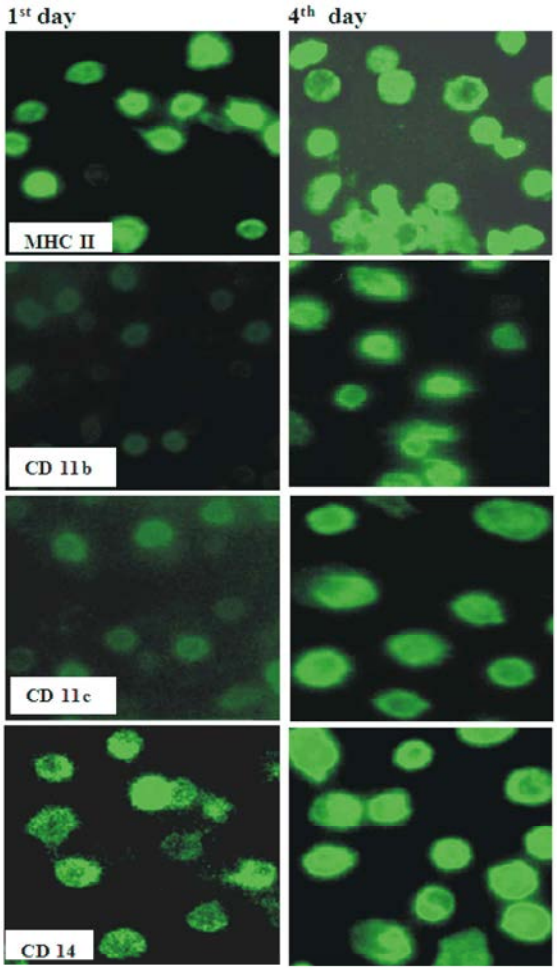


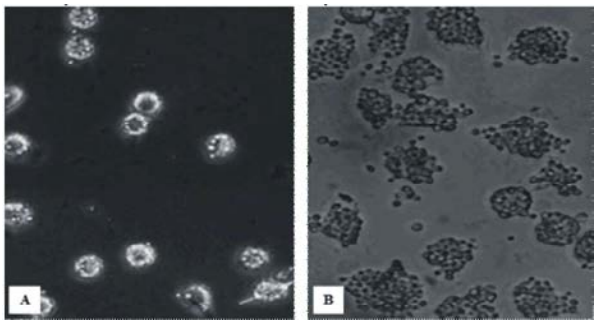
Photo 2: Surface markers expression on monocytes and MDM. Cells were separated (day 1) and cultured for 4 days and then, surface markers expression was assessed by IFA as described. The markers expression was of low fluorescent density at the 1st day of culture for both CD11b and CD 11c and increased by the 4th day of culture. The MHC II and CD14 expression level was relatively high at both the 1st day and the 4th day of culture.

$p < 0.05$ in cell phagocytic capacity to engulf ≥25, or ≥ 40 labeled yeast/cell at the 1st and 4th day of culture. On the 1st day (monocytes) of culture, the phagocytic capacity percentage of cell contained ≥ 25, or ≥ 40 was 41.0±5.0% and 13.9±0.7 %, respectively, compared to 98.9±0.7% and 97.7±0.8 %, respectively at the 4th day (MDM) of culture (Table 2). Cells phagocytic capacity was associated with remarkable increase in MDM size compared to monocytes, which showed minor increase in size as shown in Photo 3 A-D.

Table 2: Phagocytic capacity of the cells at the first and fourth day of culture

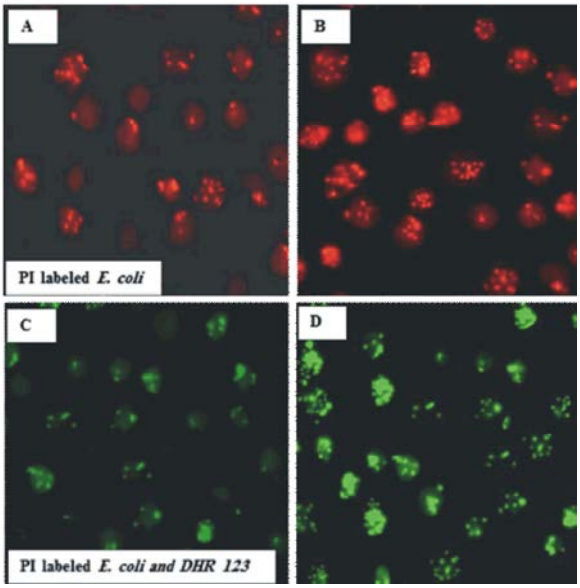
	Day of culture	
Yeast/phagocytic cells	1st day	4th day
≥ 10	92.8±4.0%	98.0±1.5%
≥ 25	41.0±5.0%	* 98.9±0.7%
≥ 40	13.9±0.7%	* 97.7±0.8

Cells from seven sheep were used in this experiment to estimate the monocytes and MDM phagocytic capacity.
*The results were significant at $P < 0.05$



1st day 4th day

Photo 3: Monocytes and MDM phagocytic capacity. The cells were separated and cultured for 3-4 days, the *C. albicans* labeled-yeast were added as described. The cells capacity to engulf yeast was higher in MDM (B) compared to monocytes (A).



1st day 4th day

Photo 4: Oxidative burst analysis of monocytes and MDM. Cells capacity to produce oxygen radicals represented by oxidation of PI labeled *E. coli* (A and B) after addition of DHR 123 to bright green color (C and D) to green (C and D) was similar in both monocytes at the 1st and the 4th day of culture.

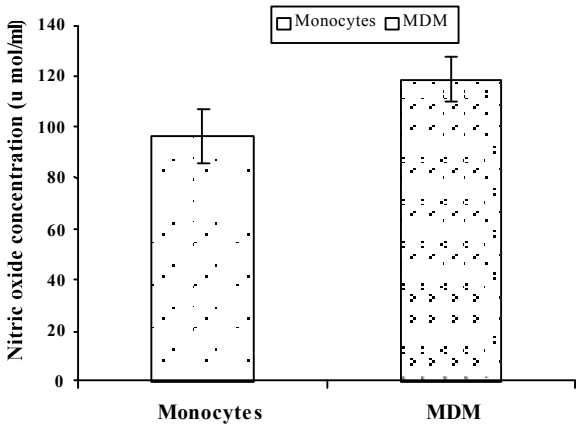


Fig. 1: Analysis of nitric oxide production by monocytes and MDM. Monocytes or monocytes-derived macrophages-exposed to LPS and nitric oxide was estimated using Griess reagent. Nitric oxide production was insignificantly different at the 4th day (MDM) compared the 1st day (monocytes) of culture. Cells from six animals were used to estimate the nitric oxide production by LPS stimulated monocytes or MDM.

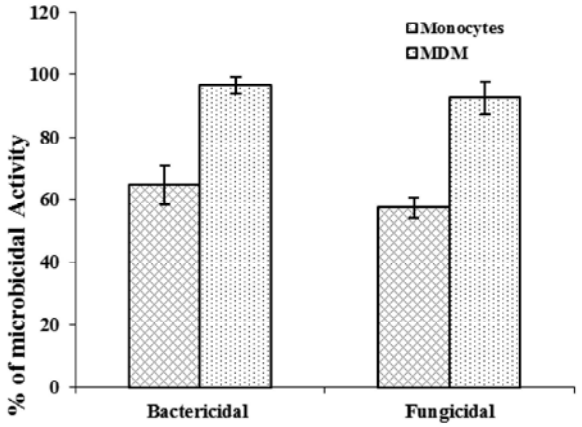


Fig. 2: Analysis of monocytes and MDM microbicidal activity. Monocytes or monocytes-derived macrophages treated with *E. coli* or *C. albicans* at day 1 (monocytes) or at day 4 (MDM). The bactericidal and fungicidal activities were significantly higher on day 4 of culture compared to day 1. Cells from eight animals were used separately to estimate the microbicidal activity of monocytes or MDM. The results were significantly different at $P < 0.05$

Oxidative Burst Activity: Oxidative burst assay was assessed in monocytes (1st day of culture) or MDM (4th day of culture). A percentage of 82.5± 4.6 % of monocytes at day 1 of culture revealed oxidative burst activity when stimulated with PI-labeled *E. coli*. Meanwhile, 98.1±1.4 % of MDM at day 4 in culture demonstrated oxidative burst activity. Oxidative burst positive cells showed bright green fluorescent, while the negative cells dull green color (Photo 4 A-D). MDM maintained this oxidative burst activity up to 30 days when the cultures were discarded (data not shown).

Nitric Oxide Production: Nitric oxide production was measured in monocytes or MDM at 12, 24 or 48 h in monocytes or MDM culture post stimulation with *E. coli* LPS. No detectable nitric oxide was detected at 12 h post stimulation. At 24 h of culture, the nitric oxide production was detectable in the culture media. Concentration of NO was 96.0±11.0 µmol/ml in monocytes culture media at 1st day of culture compared to 119.0± 8.9 µmol/ml in MDM culture media (Figure 1). No additional significance increase in nitric oxide concentration was detected at 48 h post LPS stimulation in both monocytes and MDM culture media.

Bactericidal and Fungicidal Activity: The microbicidal activity (against *E. coli*, or *C. albicans* was determined on days 1-and day 4 of culture. The cells had lower microbicidal activity on day 1 of culture for both bacteria (64.7± 6.3 %) and yeast (57.3± 2.7 %) (Figure 2). The cell microbicidal activity significantly increased day by the 4th day of culture. The killing activity increased and reached the maximum for both bacteria (96.4±3.3 %) and yeast (92.5±5.3 %) at the 4th day of culture (Figure 2).

DISCUSSION

Macrophages play critical role in both innate and acquired immune response. Obtaining and enrichment of tissue macrophages is difficult and laborious. In these studies, we have described a simple reproducible method for obtaining a high yield of macrophages in a short period of time (3-4 days). The generated macrophages were found to have typical morphologic, phenotypic and functional characteristics of tissue macrophages including cell size and cytoplasmic granules, surface markers (MHCI, MHC II, CD11b, CD11c and CD14) and ability to phogocytize microbes. The cells were also able to produce NO, reactive oxygen, cytokines and to kill intracellular organisms as confirmed in previous studies by [20-24] in human, laboratory animals, pig and bovine models. The macrophages generated by this method can be used to study macrophage-pathogen interaction and for vaccine delivery.

The results obtained in this study confirm the high enrichment in monocytes as reported by [25] for separation of human monocytes and by [9] for separation of bovine monocytes. The advantages of current method over these two previous studies were: 1. the higher cell yield at pH 6.7; 2. the ease detachment of the cells in the first days of culture and 3. economic costs, where autologous plasma gave high monocytes number with high viability compared to the more costly commercial bovine fibronectin (FN) reported by Goddeeris *et al*.[9]. However, these studies mainly studied the enrichment in monocytes culture and they neither focused on macrophages enrichment nor in their development.

In this study, a gelatin/plasma coated surfaces method resulted in high recovery of intact monocytes and ease of detachment for functional studies. Different pHs were tested and pH 6.7 was found to be the best for obtaining a high yield of monocytes and deriving maturation of monocytes to macrophages in a short time period (data not shown). Plasma is the main source for FN and freshly separated autologous plasma was a better source for FN because gelatin treated either with serum or commercial bovine FN did not result in high monocyte enrichment (unpublished data). This high yield on gelatin/plasma-coated surfaces may be due to binding of monocytes-FN receptors with the FN present in the ovine plasma. The FN/FN receptor binding is a calcium and magnesium-dependent process [7]. This may explain why the yield and the enrichment were higher in medium 199 that contained higher calcium and magnesium concentration compared to RPMI 1640 media (unpublished data).

Serum contains several growth and differentiation factors. Autocrine cell factor(s), serum growth factor(s), hormones, or cytokines (GM-CSF, M-CSF, IL-1) and other unidentified proteins may be behind the monocytes differentiation and maturation to macrophages [26-28]. The autologous serum was used at concentrations 5 %, 10 % and 15 %. No significant difference was found between the three concentrations (data not shown) and each of these concentrations was able to derive monocytes to macrophages in a short time period. In contrast, different culture conditions have been used for pig and human monocytes [20, 23] and they found that the use of antibiotics and 30% heat inactivated fetal calf serum were important to derive human and pig monocytes to macrophages rather than autologous sera. This may be due to the species difference and the used serum concentration.

Changes in the cells morphology; surface marker expression level; and microbicidal activities were taken as hallmarks to indicate macrophage maturation. In this study, morphological and functional changes were similar to other studies in human, mice and bovine macrophage system [21, 24, 27, 29] except for their earlier differentiation of the monocytes to macrophages that was prominent in this study. MDM were 99-100% of typical macrophage morphology by day 3-4 in culture. MDM were higher than 90 % positive for MHC I, CD11b and CD14 after culture in autologous serum and slightly acidic media. Phagocytic capacity, oxidative burst, nitric oxide production and microbicidal activities were dramatically increased starting from day 1. Almost 100% of the cells were positive for these activities.

In conclusion, culturing of monocytes with autologous serum at concentration of 5%, slightly acidic pH, antibiotics and on gelatin/autologous plasma coated surfaces results in a high yield of monocytes that differentiated to MDM by day 3-4 in culture and are useful in producing cells for long-term culture. This culture system also provides inexpensive method of generating high number of sheep peripheral blood monocytes-derived-macrophages compared to the other methods. We have used this system also for generation of equine, pig and gaur macrophages (data not shown). Since macrophages are the target for many viruses or intracellular bacteria, this MDM model is appropriate for studying pathogen-macrophage interactions in sheep diseases. This culture system also will be a useful tool for further *in vitro* studies to characterize the role played by MDM in cellular and humoral immune responses in sheep. Further studies are required to investigate the molecular features of the monocytes differentiation using these culture conditions.

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