Improving the methods for isolation of monocyte and establishing macrophage cell culture in caprine model

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Abstract Monocytes are widely used for immunological research, especially in the study of innate immune system. Although methods for isolation of human monocytes have been established, the procedure for non-human monocyte has not been well developed. This paper describes an improved method for isolation of monocyte and the subsequent macrophage cultivation from caprine blood. Monocytes were isolated from 16 ml of heparinized caprine blood using double density methods; the Ficoll and Percoll. The number of monocytes obtained was $5.12 \pm 0.89 \times 10^7$ cells/ml at 70 % purity. The isolated monocytes were maintained

in 10 % fetal bovine serum-enriched Dulbecco's Modified Eagle Medium for maturation to form macrophage cell culture. At the end of the experiment, the harvested macrophage was $2.48 \pm 0.33 \times 10^6$ cells/ml.

Keywords Monocyte · Macrophage · Isolation · Cell line · Caprine

Introduction

Monocytes are mononuclear cells with kidney-shaped nucleus that are larger than lymphocytes. They are part of the innate immune system, also known as circulating immature macrophages that constitute 5 % of the total leukocytes (Tizard 2000). Besides macrophages, monocytes can mature to become dendritic cells, which are important in triggering primary immune response (Banchereau et al. 2000). During infection, monocytes are triggered rapidly by the microbial stimuli and secrete cytokines, chemokines and anti-microbial factors (Serbina et al. 2008).

Macrophages are phagocytic cells, which originated from monocytes that engaged to tissues in response to inflammation or infection (Murray and Wynn 2011). The macrophages play important roles in innate and adaptive immune systems. The main function is to phagocytose pathogens as well as cellular debris but they are also capable of stimulating lymphocytes and other cells in response to the pathogen invasion.

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Various *in vitro* studies involving monocytes have been carried out on various aspects, especially in the study of intracellular parasites (Menezes et al. 2008), infection and zoonotic diseases (Arenas et al. 2000) and in oncology studies (Rey-Giraud et al. 2012). These *in vitro* studies serve as preliminary indicators due to their safety reasons and the ability to reduce uncontrollable factors that may impact the results. Thus, *in vitro* studies using monocyte and macrophage are crucial elements in understanding the pathogenesis of diseases and the immune response.

Bennett and Breit (1994) had isolated the monocytes by adhesion of peripheral blood. However, the technique often resulted in unsatisfactory outcomes when more lymphocytes were harvested (Haskill et al. 1988). Therefore, studies to improve the technique were carried out, particularly in humans and bovine models (Repnik et al. 2003; Macedo et al. 2013). So far, the monocyte isolation method has not been established for caprine blood. Furthermore, many studies using macrophage employed the commercial murine macrophages to mimic the situation in real hosts. Although this is generally acceptable, it may differ from the response by the natural host. Currently, commercially available kits are developed for human study and the methods need to be modified for animal use. Therefore, the aim of this study is to establish a simple method for isolation of monocyte that subsequently can be used to develop macrophage cell culture in caprine model.

Methods

Monocyte isolation

The method used for isolation of monocyte was modified from Repnik et al. (2003). 16 ml of blood were collected in two separate disodium ethylenediamine tetraacetic acid EDTA vacutainer tubes (Becton–Dickinson, Franklin Lakes, NJ, USA) and centrifuged in swinging bucket rotor system centrifuge (Heraeus Multifuge 3SR, Hanau, Germany) at 4,600 rpm, 4 °C for 10 min. The buffy coats from both tubes were collected and mixed with 4 ml of Rosewell Park Memorial Institute (RPMI, GIBCO, Paisley, UK) medium before being layered onto 2.5 ml of cold Ficoll (Ficoll-Paque PLUS, GE Healthcare, Little Chalfont Buckinghamshire, UK) with density of 1.070 g/ml. The suspension was centrifuged at 950 g, 4 °C for 15 min before the peripheral blood

mononuclear cells (PBMC), which interfaced between Ficoll and plasma-medium layer was collected. The cells were then layered over 3 ml of cold hyperosmotic Percoll (Sigma, St. Louis, MO, USA) with density 1.064 g/ml before centrifugation at 580g, 4 °C for 15 min. The hyperosmotic Percoll was prepared as follow: 2.43 ml Percoll mixed with 2.08 ml distilled water and 0.5 ml of 1.6 M NaCl. The monocyte layer, which appeared cloudy and occasionally with few red spots (Fig. 1) was collected and mixed with lysis buffer, and kept at 4 °C for 15 min. The lysis buffer was prepared by mixing 8.3 g NH_4Cl , 1.0 g KHCO_3 and 0.037 g EDTA in 1 l distilled water and was adjusted to pH 7.4. The suspension was resuspended gently before centrifugation at 400g, 4 °C for 15 min.

Tests for monocyte recovery, viability and purity

Following centrifugation, the supernatant was discarded and the pellet was resuspended in 1 ml Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, UK), with the following specifications: L-glutamine, high glucose with phenol red indicator, no HEPES and no sodium pyruvate (Catalogue number: 11965-092). A drop of the cell suspension was used to prepare smear on glass slide and was stained with Wright's stain for morphology confirmation. Cell count was performed using standard haemocytometer by mixing 10 μl of the cell suspensions with 190 μl of

Fig. 1 Schematic drawing of the cellular and solution fractions after centrifugation of the caprine blood sample

Fig. 2 A monocyte. Wright's stain, $\times 600$

2 % acetic acid. Only monocytes were counted, which were identified by their indented nucleus (Fig. 2). At the same time, cell purity test was performed by differential counting of neutrophils against non-neutrophilic cells.

The viability of the monocyte was determined using the trypan blue exclusion test (Phillips 1973). Briefly, the cell suspension was mixed with 0.4 % trypan blue at 10:1 ratio. Cells that excluded the trypan blue appeared transparent and were considered viable cells while dead cells were stained blue.

Cultivation of monocytes for macrophage cell culture

The harvested monocytes were resuspended in a medium consisted of 8 ml of DMEM with 10 % inactivated fetal bovine serum (FBS) (GIBCO) and supplemented with penicillin (Sigma) 100 U/ml to achieve a concentration of 1×10^6 cells/ml. The cell suspension was then transferred into 25 ml cell culture flasks and incubated at 37 °C in CO₂ incubator for 24 h. The next day after seeding, the non-adherent cells were removed and the medium was replaced by 8 ml of DMEM medium supplemented with 10 % inactivated FBS per flask. The cell cultures were maintained for at least 14 days and the medium was changed every 4 days.

Macrophage harvest

The monocyte-derived macrophages were pre-treated with trypsin/EDTA (GIBCO) in CO₂ incubator for

15 min before the cell layer was detached using a cell scraper. The cells were resuspended in DMEM medium for further use. A drop of the cell suspension was used to prepare smear on glass slide and was stained with Wright's stain for morphology confirmation. The entire procedure was repeated for at least 15 times to ensure reproducibility.

Results

Table 1 summarises the outcomes of monocyte isolation and macrophage harvest. The number of monocytes obtained from this experiment was $5.12 \pm 0.89 \times 10^7$ cells/ml, while the purity was 70 %. The trypan blue exclusion test demonstrated 90 % cell viability. The monocyte enriched layer was collected and stained with Wright's stain for the initiation of cell culture (Fig. 3).

Caprine monocytes took between 12 and 16 days of incubation to mature. Initially, the macrophages were sparsely distributed and were concentrating mainly at the edge of the flask (Fig. 4). Homogenous cytoplasmic organelles and dense granules were observed from day 7 onwards while the cell diameter started to increase. Cytoplasmic projections, known as pseudopodia was

Table 1 Monocyte isolations and macrophage harvests following each replication of the procedure

Replicate no.	Monocyte ($\times 10^7$ cells/ml)	Macrophage ($\times 10^6$ cells/ml)
1	4.73	2.27
2	4.82	2.95
3	5.01	2.76
4	3.77	2.23
5	6.36	2.21
6	6.98	1.87
7	5.55	2.65
8	4.63	2.34
9	6.17	2.40
10	4.79	2.79
11	4.98	1.99
12	4.98	2.62
13	3.99	2.71
14	5.81	2.49
15	4.29	2.97
Average	5.12 ± 0.89	2.48 ± 0.33

Fig. 3 Monocyte cell culture at day 0. $\times 200$

Fig. 4 Monocytes (black right-pointing pointer) and macrophages (right arrow) are sparsely distributed in cell culture flask. Day-6. $\times 200$

observed from day 6. On day 16, almost all macrophages demonstrated pseudopodia and were found to be compacted (Fig. 5). Therefore, cell passages were done to increase space and reduce nutrient competition. Following detachment during harvesting, the cells appeared rounded (Fig. 6). This study was able to harvest $2.48 \pm 0.33 \times 10^6$ of cultivated macrophage cells/ml.

Discussion

This paper describes a modification of the method reported by Repnik et al. (2003) on isolation of monocytes for small scale isolation and culture of

Fig. 5 Macrophages (right arrow) demonstrate the presence of pseudopodia and appear to be compacted. Day-16. $\times 400$

Fig. 6 The macrophages. Wright's stain. $\times 600$

monocytes from caprine blood. This method used a standard swing out centrifugation machine and required between 90 and 120 min to complete. The procedure offers the advantage of reproducibility and consistent outcome as the procedure had been repeated for up to 50 times with relatively consistent results. The purity of monocytes was 70 %, which is equivalent to Repnik et al. (2003). Many other studies used larger volumes of blood (Almeida et al. 2000), which restricted the application on caprine model. According to Johnson et al. (1977), the important factors that improved cell survival were the choice of culture medium and the use of plastic culture wells. The current study proved that DMEM medium is a good culture medium for caprine model.

Monocytes, known to have the lightest density compared to other cells (Feige et al. 1982) can be

isolated through the double density method. The Ficoll solution at concentration of 1.070 g/ml was used to separate PBMC from the blood. This technique was reported in several studies (Gower et al. 2002; Stoffregen et al. 2013), which were successfully used in the PBMC isolation. This layer is important to be isolated as a preliminary step before monocyte can be separated.

Percoll, with a density of 1.064 g/ml is a good medium to be used in monocyte isolation. This medium was useful for caprine blood although the product was manufactured for human purposes. Thus, the capability to reduce lymphocyte contamination requires less amount of blood to be used.

In this procedure, some erythrocytes might contaminate the monocyte-enriched layer. This problem could be solved by incubating the suspension with lysis buffer for 10 min to lyse the red blood cell. Three washing steps should be performed after the incubation period to ensure the lysis buffer was totally removed.

The monocytes should be observed daily with an inverted phase contrast microscope at magnification 100–200×. The FBS enriched DMEM was required to be freshly prepared before use to reduce contamination. During spontaneous maturing into macrophages, the cells were observed to ‘anchore’ at the edge of the flask. During harvesting, chemical and physical methods were combined to detach macrophages from the flasks due to the morphology of macrophages that have ‘arm-like’ structure.

Finally, the monocyte isolation can be improved by double density gradients as shown by Flusk (1981) and Boyum (1983). In conclusion, the method described here is simple and reproducible for monocyte isolation and macrophage cell culture of caprine model.

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Conflict of interest The authors have declared no conflict of interest.

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