Macrophages play an important role in host defense, in both innate and acquired immunity [19]. They are also important in immune responses, such as ingesting abnormal cells, cell debris, or normal cells, as well as activating the T-cell response observed in various tumors [1] or autoimmune diseases [6]. Recently, a lot of attention is being paid to macrophages because of the theory of macrophage polarization into classically activated M1 and alternatively activated immunomodulatory M2 cells [2].

Few reports have focused on the cellular function of canine macrophages, possibly because canine macrophages are difficult to isolate and culture in vitro. Macrophages can be obtained from bronchoalveolar lavage fluid [18], bone marrow [20], and peripheral blood [3, 8, 10, 16], and cultured as adherent cells on plastic plates with or without cytokine addition. Among the above-mentioned macrophage sources, the most accessible source is peripheral blood, since it does not require anesthesia for sample collection. However, the number of macrophages obtained from peripheral monocytes is relatively low. Using the method reported by Bueno et al. [3], approximately 1% of PBMCs cultured in the absence of cytokines gives rise to macrophages. This suggests that 60 ml of peripheral blood would be required to obtain approximately 4 × 10^5 macrophages. A modification of this method has been made by others [16]. By adding granulocyte-macrophage colony stimulating factor (GM-CSF) and replacing FBS for dog serum, Sampao et al. [16] were able to improve cell survival. The exact difference was not described. In order to investigate the various functions of macrophages in the dog, it would be of great interest to obtain macrophages from dogs with various diseases. Since it would be difficult to collect a large volume of peripheral blood from diseased dogs, a more efficient method would be necessary to reduce the blood sampling volume. Moreover, since more than 10 days of cultivation are required in all previously reported methods [3, 8, 10, 16], a shorter cultivation period would be more convenient for investigation. The aim of this study is to establish a simple and rapid method to obtain macrophages from small volumes of dog peripheral blood.

MATERIALS AND METHODS

Isolation of PBMCs from healthy dogs: PBMCs were obtained from 5–6-year-old healthy beagles using an ordinary gravity sedimentation method. Auffy coat obtained from EDTA-treated peripheral blood was resuspended in PBS, overlaid on Optiprep (AXIS-SHIELD, Oslo, Norway) adjusted to the density of 1.079, and centrifuged at 800 g for 30 min at room temperature. Isolated PBMCs were resuspended in PBS and overlaid on Optiprep adjusted to the density of 1.063. Contaminating platelets were removed by centrifugation at 500 × g for 15 min at room temperature. Using this method, 9–20% of the obtained PBMCs were monocytes, as assessed by flow cytometry (data not shown).

Cell culture: PBMCs were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% FCS (Biowest, Nuaille, France) and antibiotics at a concentration of 1 × 10^6 cells/ml. Two milliliters of PBMCs (2 × 10^6 cells) was plated on a 35-mm tissue culture-treated dish (Corning, Lowell, MA, U.S.A.), stimulated with 10 ng/ml PMA (Sigma-Aldrich, St. Louis, MO, U.S.A.), and cultured at 37 ºC with 5% CO₂. Monocytes were allowed to attach to the plastic dish for 1 day and then washed with culture
medium. Non-adherent cells were discarded. Two milliliters of culture medium containing 10 ng/ml PMA was added to the culture dish to induce macrophage differentiation for further 6 days, 7 days in total. The cultivation period will be stated as days after the beginning of PBMC stimulation.

For morphological observation, adherent cells were harvested with a cell lifter (Corning). Slides with a monolayer of cells were prepared using Cytospin 4 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The monocyte population was distinguished from the lymphocyte population at day 0 (before stimulation with PMA) using the FlowJo software (Tree Star, Ashland, OR, U.S.A.). A mouse IgG1 isotype control was used as primary conjugate was prepared according to the manufacturer’s specifications and transferred to the culture dishes. Cells were incubated for 2 hr at 37°C with the suspension, washed twice, and subjected to morphological observation.

The phagocytic activity was also determined using fluorogenic dye-conjugated bacteria particles (pHrodo E. coli BioParticles conjugate; Invitrogen, Carlsbad, CA, U.S.A.). The fluorescent intensity of pHrodo particles increases dramatically as the pH of its surroundings becomes more acidic upon phagocytosis. A suspension of pHrodo BioParticles conjugate was prepared according to the manufacturer’s instructions and transferred to the culture dishes. Cells were incubated for 2 hr at 37°C with the suspension, washed twice, and analyzed by flow cytometry.

Flow cytometric analysis: Cell suspensions were washed with staining medium (PBS supplemented with 5% FCS) and stained with the following mAbs for 30 min at 4°C (all mAbs except anti-CD14 were obtained from the Leukocyte Antigen Laboratory, Davis, CA, U.S.A.): anti-CD14 (TUK4; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-CD11b (CA16.3E10), anti-CD11c (CA11.6A1), anti-CD11d (CA11.8H2), anti-MHC II (CA2.1C12), and anti-CD1a (CA13.9H11). Then, cells were washed twice with staining medium and stained with FITC-conjugated anti-mouse IgG, or IgG2 for another 30 min at 4°C. Cells were washed twice and analyzed with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, U.S.A.). A mouse IgG1 isotype control was used as primary antibody to analyze the background fluorescence intensity at each time point. The obtained data was analyzed using the FlowJo software (Tree Star, Ashland, OR, U.S.A.). The monocyte population was distinguished from the lymphocyte population at day 0 (before stimulation with PMA) based on its light scatter properties. After stimulation with PMA, all the adherent cells were gated to exclude cell debris.

RESULTS

Morphological changes of isolated cells during cultivation: An abundant number of round-cells were attached to the culture dish 1 day after stimulation. These cells were classified as monocytes and gradually increased in size during the 7-day cultivation period, as also confirmed by the light scatter plot obtained in the flow cytometer (Fig. 3A). Five days after stimulation, these cells presented irregular to round-shaped nuclei and vacuolated cytoplasms (Fig. 1A and 1B), and stained positively for esterase (Fig. 1C). Coincidentally, multinucleated cells with 2 or 3 nuclei began to appear sometimes with large size.

Evaluation of phagocytic activity: The phagocytic activity of differentiated macrophages was determined by adding latex beads to the cultures 7 days post stimulation. After incubation, all the observed adherent cells were engulfing latex beads. Some of them were filled with latex beads (Fig. 2A).

The phagocytic activity was further evaluated using fluorogenic dye-conjugated bacteria particles. The fluorescence intensity of the conjugated dye pHrodo increased with phagocytosis-induced acidification. As shown in Fig. 2B, the fluorescence intensity of pHrodo increased in nearly all the cells after 5 days of cultivation, confirming their high phagocytic activity.

Taken together, these PBMC-derived cells are adhesive to plastic dishes, increase in size over cultivation time, and possess phagocytic activity. In addition, they exhibit multiple nuclei. Thus, we concluded that these cells are monocyte-derived macrophages.

Evaluation of the macrophage yield obtained from PBMCs: We calculated the yield of macrophages obtained from PBMCs isolated from 3 different dogs. The percentage of monocytes was calculated based on the number of CD14-positive cells present before stimulation with PMA. After 5 days of cultivation, cells were scraped off from the dish and counted using a hematocytometer. The PBMCs from dogs A, B, and C contained 9, 20, and 19% monocytes before stimulation, respectively. This indicates that 1 culture dish with 2 × 10^6 PBMCs from dogs A, B, and C contained 1.8 × 10^5, 4.0 × 10^5, and 3.8 × 10^5 monocytes, respectively. Five days of culture with PMA resulted in 2.5 × 10^5, 1.2 × 10^5, and 1.2 × 10^5 viable macrophages from dogs A, B, and C, respectively. Since more than 2 × 10^5 PBMCs can be routinely isolated from 20 ml of peripheral blood, it would be possible to reduce the volume of blood sampling depending on the required number of macrophages. In contrast, adherent cells were rarely observed from PBMC without PMA addition. We also examined the effect of GM-CSF by adding 20 µg/ml of feline recombinant GM-CSF (fGM-CSF) with or without PMA. No difference in adherent cell yield was observed by fGM-CSF addition.

Changes in cell surface antigen expression: Expression of cell surface antigens was evaluated by flow cytometry (Fig. 3). At day 0 (before stimulation with PMA), the monocytes were negative for CD1c and CD11d but largely positive for MHC II, CD14, CD11b, and CD11c. However, the expression of MHC II, CD11b, and CD11c was dramatically reduced to be almost negative after 5 days of stimulation, while the expression of CD1c and CD11d remained negative. Expression of CD14 was upregulated and kept to be strongly positive during the 5 days of cultivation.
In this report, we established a simple and rapid method to obtain macrophages from canine peripheral blood. PMA is used to induce macrophage differentiation in several human monocytic cell lines. THP-1 (human monocytic leukemia cell line), HL-60 (human promyelocytic leukemia cell line) and U937 (human histiocytic lymphoma cell line) change their characters including morphology and phagocytic activity from monocyte-like rather into macrophage-like [17, 21, 23, 25]. Human PBMC-derived monocytes were also reported to differentiate into macrophage, although morphological and functional analysis was not performed [23].

These cells exhibited multiple nuclei, which is also one of the characters of macrophages. Human multinucleated giant cells are believed to derive from monocyte-derived macrophages by fusion in a variety of inflammatory condi-
It is of note that nearly all of the macrophages showed active phagocytosis 5 days post stimulation. Previous reports required more than 10 days of cultivation for macrophage maturation [3, 8, 10, 16]. Shorter cultivation period would be more convenient for investigation.

Further, the macrophage yield seemed to be much higher than that reported previously. Nonetheless, the accurate figures of the obtained macrophages were not mentioned in many of these reports [8, 10, 16]. In a previous study, $4.1 \times 10^5$ macrophages could be obtained from about $3.7 \times 10^7$ PBMCs (mean value from 22 dogs), which was about 8 times less than what we obtained [3], as concluded in Table 1. In this report, the authors also pointed out the low correlation between the peripheral blood monocyte counts and the resulting macrophage counts. This is consistent with our data, although we have studied a reduced number of dogs.

Macrophages and dendritic cells (DCs) are the 2 major populations that differentiate from peripheral monocytes. The cell surface antigen expression of DCs was reported in two independent studies in which these cells were obtained from peripheral blood. One report has showed that expression of MHC II and CD1a increases during maturation toward a DC phenotype [22]. Another report also showed high expression of MHC II [24]. On the contrary, the cell
surface antigen profile of peripheral monocyte-derived macrophages has been scarcely reported. One report using immunohistochemistry showed that macrophages in the BM and spleen specifically express CD11d, whereas monocytes in the BM do not [14].

The macrophages obtained by our method were positive for CD14; weakly positive for CD11b, CD11c, and MHC II; and negative for CD1c and CD11d. CD14 is a glycoprotein that directly binds to LPS. It is commonly used as a monocyte lineage marker in various species including the dog [3, 5, 22]. Expression of CD14 clearly distinguishes these cells from granulocytes or lymphocytes. Further, low expression of MHC II indicates that these cells are macrophages rather than DCs. Low MHC II expression on macrophages are of MHC II indicates that these cells are macrophages rather from granulocytes or lymphocytes. Further, low expression 5, 22].

Expression of CD14 clearly distinguishes these cells in the cyte lineage marker in various species including the dog [3, 5, 22]. Expression of CD14 clearly distinguishes these cells from granulocytes or lymphocytes. Further, low expression of MHC II indicates that these cells are macrophages rather than DCs. Low MHC II expression on macrophages are also consistent with human and mouse compared to that on DCs [12, 19]. However, this result is not in agreement with a previous report in which spleen-derived macrophages were CD11d-positive [14]. The cell surface antigen expression of macrophages derived from peripheral monocytes in vitro has not been previously studied. It is possible that the surface antigen expression of macrophages derived from peripheral monocyte in vitro and resident spleen macrophages is different as indicated in humans [15]. Interestingly, the expression of MHC II was nearly negative in this study. Although we did not investigate antigen presentation in these cells, it is conceivable that they are weak APCs. Recent reports showed that the expression of MHC II is downregulated in macrophages with M2 phenotype that have suppressive effects on T-cell activation [4, 11]. It would be interesting to determine whether the cells obtained in our study have M2 phenotype.

We extended the cultivation period up to 12 days. However, the cell surface antigen profile did not differ from that at day 5 (data not shown). Taking into account that macrophages cultured for 5 days post-stimulation showed phagocytic activity, we consider that the macrophages can be used for experimental assays after only 5 days.

In conclusion, we established a simple and rapid method to obtain a large amount of macrophages from canine peripheral blood. The obtained macrophages exhibited a typical macrophage phenotype, as judged by their surface antigen expression (with the exception of CD11d) and phagocytic activity. Importantly, the cultivation period used in our method is relatively short (5 days) in comparison to other methods previously reported (10 days or more). This method could be useful for the study of various canine diseases characterized by macrophage/monocyte activation.

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**REFERENCES**


**Table 1. Comparison of macrophage yield efficiency obtained from PBMCs**

<table>
<thead>
<tr>
<th>Source</th>
<th>Stimulation</th>
<th>Macrophage count obtained from 10⁷ PBMCs</th>
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<td>PBMC from healthy dog</td>
<td>PMA 10 ng/ml</td>
<td>8.0 × 10⁴</td>
<td>this study</td>
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<tr>
<td>PBMC from healthy dog</td>
<td>none</td>
<td>1.1 × 10⁵</td>
<td>[3]</td>
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