

An *in vitro* monocyte culture method and establishment of a human monocytic cell line (K63)

Katsuyuki Kadoi

Summary

A novel method of monocyte culture *in vitro* was developed. The fraction of monocytes was obtained by density centrifugation of heparinised human venous blood samples. Monocytes were suspended in a modified Rosewell Park Memorial Institute medium (RPMI)-1640 (mRPMI) supplemented with 10% non-inactivated autologous serum added to the feeder cells. An avian cell line was used for feeder cells. Only those monocytes that settled on feeder cells grew rapidly at 37°C-38°C into a formation of clumped masses within two to three days. The cell mass was harvested and subcultures were made without feeder cells. A stable cell line (K63) was established from subcultures using a limited dilution method and cell cloning in microplates. K63 cells were adapted for later growth in the mRPMI medium supplemented with 10% foetal calf serum. The cells were well maintained at over 50th passage levels. This method proved to be applicable for monocyte cultures of animals as well.

Keywords

Cell line, Culture, *In vitro*, K63 cell line, Monocyte.

Metodo di coltura monocitica *in vitro* e costituzione di una linea cellulare monocitica umana (K63)

Riassunto

È stato sviluppato un metodo innovativo di coltura monocitica *in vitro*. La frazione di monociti (Mo) è stata ottenuta mediante centrifugazione in gradiente di densità da campioni di sangue venoso umano eparinizzato. I Mo sono stati messi in sospensione in terreno Rosewell Park Memorial Institute (RPMI)-1640 modificato (mRPMI), arricchiti con siero autologo non inattivato al 10% e aggiunto su cellule feeder. Per le cellule feeder è stata utilizzata una linea cellulare aviaria. Solo i monociti insediati sulle cellule feeder sono cresciuti rapidamente a 37-38°C formando masse agglomerate in due o tre giorni. Sono stati raccolti gli agglomerati cellulari e sono state effettuate subculture senza cellule feeder. Partendo dalle subculture è stata istituita una linea cellulare stabile (K63) utilizzando un metodo di diluizione limitata e la clonazione cellulare in micropiastre. Le cellule K63 sono state adattate per l'accrescimento differito in terreno mRPMI integrato con siero fetale di vitello al 10%. Le cellule sono state adeguatamente preservate oltre il livello del 50^{esimo} passaggio. Questo metodo si è dimostrato applicabile anche alle colture di monocitici di animali.

Parole chiave

Coltura, *In vitro*, Linea cellulare, Linea cellulare K63, Monociti.

Laboratory of Veterinary Microbiology, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan
katsuyukikadoi@yahoo.co.jp

Introduction

The monocyte-macrophage lineage has been considered as a pivotal role in immunology (18). In addition, these cells play a key role in various diseases, including atherosclerosis, sepsis, cancer, tuberculosis and human immunodeficiency virus 1 (HIV-1) (4, 5). In recent years, these cells have been recognised as targets for several viral infections in humans and animals. Consequently, the method of cultivation of these cells *in vitro* is urgently needed. Several methods have been developed to generate human primary monocytes and macrophages for *in vitro* studies but methods differ in cell yield and application for animals (4). A novel and less costly method has been developed for *in vitro* culture of monocytes. It was not necessary to add extra growth factors, such as macrophage-colony stimulating factors (M-CSF) and other cytokines including interleukin-1, -3, and -6. However, feeder cells were essential solely for the primary culture stage. The method can be reproduced with ease not only for human monocytes but also for monocytes from animal blood.

Materials and methods

Basal medium

The basal medium for monocyte culture is a modified Rosewell Park Memorial Institute medium 1640 (RPMI-1640). The original RPMI-1640 medium contained 1 g of glucose per 1 000 ml. Glucose was added to make 2 g per 1 000 ml. Galactose and fructose were also added at 0.5 g per 1 000 ml, respectively. These are the points of modification. Antibiotics were incorporated, namely: kanamycin sulphate, streptomycin sulphate and Na ampicillin. All ingredients were dissolved in pure water (Elix & RiOs systems, Nihon Millipore K.K. Tokyo) without the inclusion of sodium bicarbonate and were filtered using a 220 nm millipore filter (Stericup, Nihon Millipore K.K., Tokyo). The sodium bicarbonate solution was prepared separately at 7.5% aqueous solution and sterilised by autoclaving. It is generally added at 2.0 ml per 100 ml of cell growth medium.

Cell growth medium, primary and subcultures

Cell growth medium for primary cultures consisted of 9 parts of mRPMI and 1 part of non-inactivated autologous serum. Sodium bicarbonate solution was added at 1.5 ml per 100 ml of growth medium for primary cultures. The part of plasma separated from original blood sample was equally applicable as autologous serum. All procedures were conducted at room temperature and most of the handling procedures were performed using a biosafety cabinet with a high efficiency particulate air (HEPA) filter. The heparinised venous blood (30 ml) was collected from a healthy man (K) who was 63 years of age, blood type A, and centrifuged at 2,000 rpm for 20 min. The part of plasma was re-centrifuged for clarification and was filtered immediately with a 220 µm membrane for the preparation of autologous plasma. The precipitated cells were diluted with an equal volume of Dulbecco phosphate buffered saline (PBS), without inclusion of Ca⁺⁺ and Mg⁺⁺ and were overlaid on an equal amount of Lymphoprep (density: 1 077 g/ml, Axis-Shield PoC AS, Oslo) in centrifuge tubes. These tubes were centrifuged at 2 500 rpm for 20 min. The whitish cellular band that formed at the middle of tubes was harvested. This fraction contained monocytes and lymphocytes with some platelets. The fraction was washed with mRPMI adjusted at neutral pH. The number of monocytes was enumerated using a haemocytometer. Monocytes are easily distinguished from others since they are much larger in size.

The monocyte suspension, containing over 10 000 cells in 20 ml of growth medium was fed onto the feeder cell layer of Kadoi's chicken embryonic kidney cell line: (KCEK) (13). The feeder cell monolayer was prepared two to three days in advance. In stationary incubation at 37°C-38°C without CO₂ flow, monocytes rapidly adhered to the feeder cells in a flattened shape. Some monocytes showed mitotic division within 5 h to 6 h incubation as shown in Figure 1. Rapid growth of monocytes in clumped cell mass (CCM) was observed on days 2 and 3 of incubation, as shown in Figure 2. They were slightly rooted onto feeder

cells with transparent collagenous material. The size of CCMs was not uniform and some consisted of hundreds of monocytes grown *in vitro* which later floated. The cells were spherical in shape at this stage and adhered to one other. These CCMs were harvested by flushing the medium with a pipette, followed by centrifugation. CCMs were harvested on the third day of primary culture and re-suspended in freshly prepared growth medium supplemented with 10% foetal calf serum (FCS). The number of individual cells was difficult to enumerate with the haemocytometer since the clumped cells were stuck to one other. The CCM suspension prepared in 40 ml of growth medium was dispensed into flasks (Nunc, Delta Surface, Roskilde) for subculturing. The feeder cells were no longer needed after primary culture.

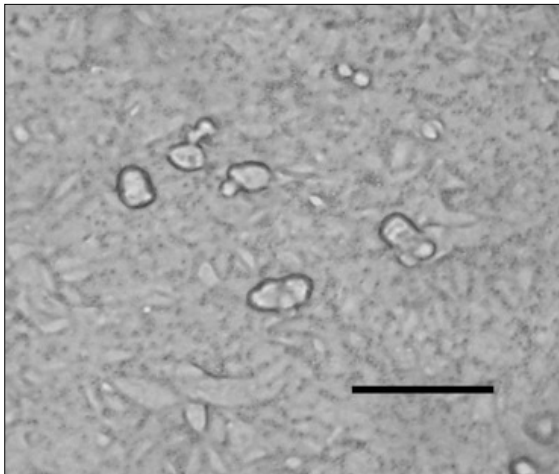


Figure 1
Primary human monocytes on feeder cells
(day 1)
Culture inspected under microscope, CK Olympus,
and photographed by Fujifilm Finepix J10
(Bar = 100 μ m)

Within two days of incubation, active outgrowth of monocytes occurred from adhered CCM as shown in Figure 3. When the cellular growth was rapid, half of the growth medium had to be refreshed every 2 days and the amount of 7.5% sodium bicarbonate solution was increased to 2 ml-2.5 ml per 100 ml of growth medium. Most CCMs were removed within one week and confluent cell layers remained for a similar period as seen in Figure 4. A small number of feeder cells

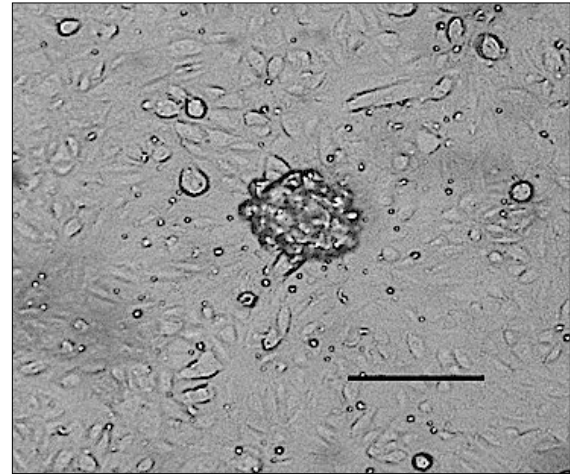


Figure 2
Primary human monocytes on feeder cells
(day 3), growth in clump formation
(Bar = 100 μ m)

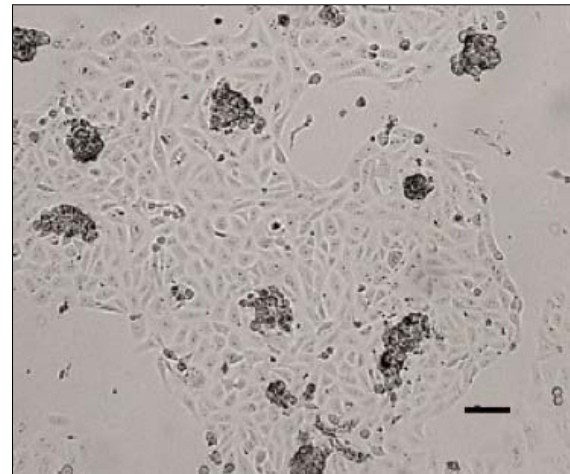


Figure 3
Growth of human monocytes started from
clumped cell mass in subculture (day 3)
(Bar = 100 μ m)

incorporated in the subculture were phagocytosed by monocytes and non-adhesive cells were removed during the refreshment of growth medium. When subcultures were almost confluent, the cells were treated with trypsin-ethylenediamine-tetraacetic acid (EDTA) solution (0.025% trypsin and 0.05% EDTA in PBS) for cell dispersion and further passages were performed. Autologous serum or autologous plasma did not need to be added to the cell growth medium during the subculture stage, but a highly qualified FCS was supplemented. Seeding with 3-4 million cells per flask (75 cm² Nunc, Roskilde), a confluent cell layer formed after 3 to 4 days of incubation

and further cell passages were made when a large amount of cells was required. In this way, over 100 million monocytes were produced in 4-5 weeks.

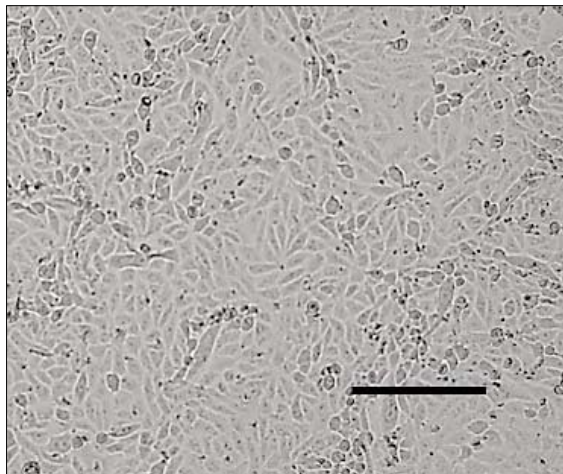


Figure 4
Confluent monolayer of subculture (day 7)
started from clumped cell mass
Clumped cells were removed by refreshment of
growth medium
(Bar = 200 μ m)

Establishment of a cell line

Since cells were adapted to growth in the medium supplemented with FCS, cell cloning was conducted by a limited dilution method and cell cloning in microplates (Nunc, 48-well type, Roskilde) (11, 12, 14). An established cell line was named K63 since the original blood material was collected from a donor K at 63 years of age. The cells were well cryopreserved at -80°C and/or in liquid nitrogen.

Karyotyping

A chromosome study of K63 cells was made at 20th, 25th and 49th passages after cloning. Cells at logarithmic phase in growth, were added to colchicine (0.02 mg/ml) and incubated further for 6 h-8 h (11, 12, 14). Karyotyping of the cells was quasidiploid and the major chromosome number detected in these passage levels was 46 (2n). It was surprising that a very similar karyotype was confirmed in the cells at the 49th passage. K63 cells retain the fundamental monocytic properties, but growth kinetics and biological

behaviour differ from those in the early stages of subculture.

Morphology

Slip inserts in Leighton tubes were prepared for cytological morphology and cytochemistry. Slip cultures were fixed in methanol and stained by Giemsa. K63 cells are pleomorphic epithelioid cells and suspected to be promonocytes (6, 7). A typical morphology is shown in Figure 5. The cellular size is variable. Young cells in a log phase growth are small and are triangular in shape, while cells cultured for more than three days become larger.

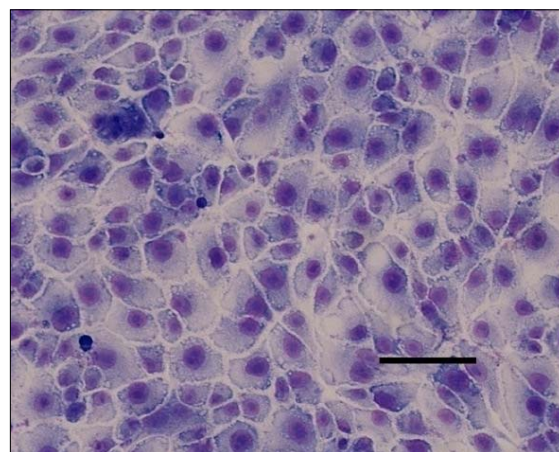


Figure 5
K63 monolayer in showing a typical epithelioid
morphology
The slip culture was fixed in methanol and stained
with Giemsa
(Bar = 100 μ m)

Cytochemistry

Two marker enzymes, generally known for monocytes and macrophages, were qualitatively examined using cytochemical methods. They were non-specific esterase (27) and acid phosphatase. Commercial kits (Muto Chemicals, Tokyo) were used. Clear positive reactions of both enzymes were demonstrated in the cytoplasmic areas of cells, as shown in Figures 6 and 7.

Phagocytosis

Phagocytic action was clearly demonstrated in the cells shortly after seeding. The inoculum of *Saccharomyces cerevisiae* was trapped and

engulfed in vesicles that formed on the cell surface and was digested following prolonged incubation in a very similar manner to that reported in an early report on canine monocytes (11). Inorganic fine particles, such as silica, hydroxyapatite and quartz sand, were enclosed in vesicles in the central part of the cytoplasm, near the nuclei. After prolonged incubation lasting approximately 7 days, some cells naturally differentiated and became very large cells. They were morphologically similar to macrophages. These cells showed more intensive positive reactions of both non-specific esterase and acid phosphatase.

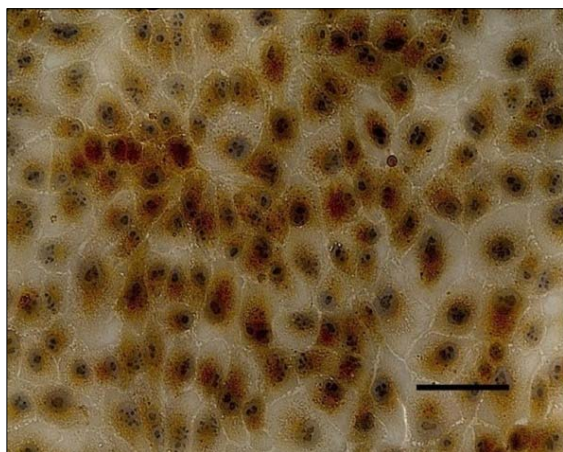


Figure 6
Non-specific esterase reaction demonstrated on K63 cells at 44th passage
Enzymes are shown by the yellow-brownish granules in the cytoplasmic area by cytochemistry
(Bar = 100 μ m)

Surface antigen expression

K63 cells at 45th-47th passage levels were analysed by flow cytometry on FACSCalibur™ (Becton Dickinson, Franklin Lakes, New Jersey) equipped with CellQuest software (Becton Dickinson). The following monoclonal antibodies (mAbs), mainly related to monocyte-macrophage, were purchased (Dako Japan Co. Ltd, Tokyo):

- anti-human CD1a (010)
- anti-human CD4 (MT310)
- anti-human CD11b (2LPM19c)
- anti-human CD11c (KB90)
- anti-human CD14 (TUK-4)
- anti-human CD16 (DJ130c)
- CD32 (KB61)

- anti-human CD34 class-I (BI-3C5)
- anti-human CD34 class-II (QBEnd 10)
- anti-human CD35 (To5)
- anti-human CD64 (10.1)
- anti-human CD68 (KP1)
- anti-human CD68 (PG-M1)
- anti-human CD91 (A2MRa-2)
- anti-human HLA-DP-DQ, DR/FITC (CR3/43).

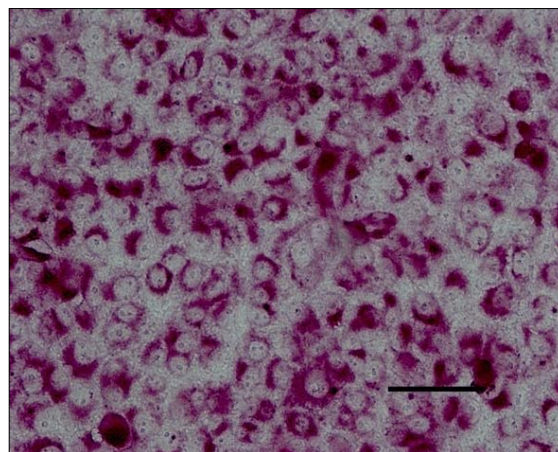


Figure 7
Acid phosphatase reaction demonstrated in cytoplasmic area of K63 cells at 45th passage
Enzymes are shown in the cytoplasmic area (reddish colour) by cytochemistry
(Bar = 100 μ m)

Secondary antibody used was anti-mouse immunoglobulin (IgG) (L+H) goat IgG fluorescein isothiocyanate (FITC)-conjugated (mAb) (Jackson ImmunoResearch Laboratories, Inc. West Grove, Pennsylvania). In preliminary tests, the cells were not reactive to most of these mAbs, except anti-human HLA-DP-DQ, DR/FITC (CR3/43). However, the cells became reactive to all mAbs mentioned above when they were pre-treated with interferon (1 000 units of rHumIFN γ) (Sigma-Aldrich, Tokyo).

Tumorigenicity

Five suckling golden hamsters, aged 5 days, were inoculated subcutaneously with 1 million K63 cells, at 12th passage level *in vitro*, per head. The hamsters were inspected over a period of three months and autopsy and histopathological examinations were performed. No tumour formations were detected.

Virus infection

Cells were permissive at least for Aujeszky's disease virus, vesicular stomatitis virus and Newcastle disease virus and infective progenies were produced in successive passages. K63 cells have a very high susceptibility at least for Aujeszky's disease virus. A characteristic CPE, as shown in Figure 8, appears within one day post infection.

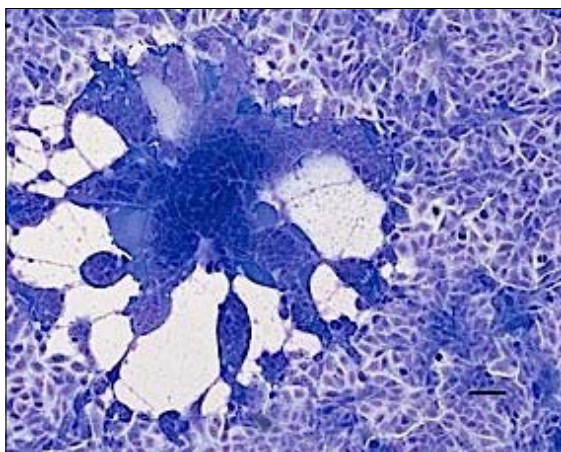


Figure 8
Characteristic cytopathic effect produced in K63 cells infected with Aujeszky's disease virus. Fusion foci started to occur on day 1 post infection. The slip culture was fixed in methanol and stained with Giemsa. (Bar = 100 μ m)

Results and discussion

The method of monocyte culture mentioned above was developed eight years ago and reproducibility was confirmed several times with venous blood samples of the same human donor and also with blood of several animal species. Prior to the development of this method, the author was able to cultivate canine, swine and feline monocytes *in vitro* without feeder cells (11, 12, 14). However, it was not easy to achieve the generation of monocytic clones *in vitro* without feeder cells. However, encouraged by these results of monocytic cell cultures of animal blood origin, seven trials were performed on the human venous blood of the same donor (K). None of human monocytes were capable of adhering to culture flasks and only negative results were obtained.

Apart from monocyte cultures, the author had been engaged in the study of hybridomas for monoclonal antibody preparation and hybridomas hardly grew when highly diluted on microplates. In the meantime, the author established two chicken embryonic cell lines with a newly prepared nutrient medium (13), although it was not published immediately due to patent approval in Japan.

In practice, hybridomas grow with ease on chicken cell lines even at high dilutions. This phenomenon suggested a possibility of these chicken cell lines as feeders for human monocyte cultures. An initial trial was performed with KCEK cells (13). Human monocytes grew readily on the feeder cells. Venous blood materials obtained from experimental animals were also examined and the results were comparable to those obtained from human monocyte culture. Once primary cultures were prepared on feeder cells, subcultures were made in the growth medium mentioned above. The growth rate was very similar to other established cell lines. At the subculture stage, the monocytes grew more rapidly than some of established epithelial cell lines. In practice, not all plastic cell culture flasks commercially available give good results. Monocytes may produce proteases (10, 25, 26). Among them the enzymes, estimated at 79 kDa and 81 kDa, may digest gelatin (22). The activity of these enzymes became evident in cultures of monocytes after more than two days. In this case, cell growth medium became slightly turbid and fine gelatinous fragments were detected upon microscopic observation in gelatin-coated flasks.

Since K63 cells, at 45th-47th passages, were tested with the panel of mAbs mentioned above, the cells were not reactive to these mAbs. Consequently, careful studies were conducted to activate K63 cells. It is generally known that interferons (α , β and γ) modulate and or activate mononuclear cell function (1, 2, 3, 8, 9, 18, 19, 23, 24). Therefore IFN- γ in the recombinant form (rIFN- γ), which is commercially available, was used in this experiment as mentioned above. Shortly after the treatment with rIFN- γ , the cells clearly reacted with all of mAbs, except mAbs against

HLA-DP-DQ, DR since the cells were reactive with the mAb without rIFN- γ treatment. It is a classical method showing that IFN- γ was an effective stimulant.

It is important to note that K63 cells can adapt to grow *in vitro* and be passaged over 40 times after cloning in the cell growth medium supplemented with FCS. The cloned cells represent a particular cell population which was adapted to grow in restricted conditions that were very different from the original physiological environment *in vivo*. It is also a well known phenomenon that fully differentiated normal human monocytes hardly grow *in vitro*. Even after an extended period, monocyte maintenance *in vitro* needs the addition of growth-stimulating factors in culture media in general.

According to early reports on the morphology of human monocytic cells (6, 7), K63 cells were estimated to be promonocytes. It was clearly demonstrated that monocytic cells maintained *in vitro* were the population of cells at an immature stage (6, 7). It was also understood that K63 cells are not fully mature cells and, therefore, they can grow easily *in vitro*. On the other hand, recent data indicate that human monocytes are possible progenitor cells with multipotential capacity (16, 17, 20, 21).

Several fundamental examinations have been performed on K63 cells although the methods and data have not been included in this paper.

K63 and also other monocytic cells showed a wide range of bactericidal functions, but not for fungi. K63 was hardly infected with a vaccine strain of *Mycobacterium tuberculosis* (BCG) when approximately 10 bacteria per cell were inoculated. Less than 1% of the cells ingested BCG. However, once the bacteria invaded, their numbers increased following incubation. This information has been presented in immunology text books. In our limited experience, primary and secondary cell cultures have a wider range of viral susceptibility than those of established cell lines. For particular objectives, such as virus assays, it is convenient to keep cellular stocks at the subculture stage. A more refined method to produce a homogenous population of human monocytes starting from the human embryonic stem cell lines has been developed (15). Biology of monocytes has become an important focus for modern cytology, immunology, pathology and virology. Informative investigations have increased but in-depth fundamental studies are required for the medical therapeutic applications.

References

1. Adams D.O. & Hamilton T.A. 1987. Molecular transductional mechanisms by which IFN- γ and other signals regulate macrophage development. *Immunol Rev*, **97**, 5-27.
2. Adams D.O. & Hamilton T.A. 1992. Molecular basis of macrophage activation: diversity and its origins. In *The natural immune system – the macrophage* (C.E. Lewis & J.O'D. McGee, eds). IRL Press, Oxford University Press, Oxford, 75-114.
3. Ball E.D., Guyre P.M., Shen L., Glynn J.M., Maliszewski C.R., Baker P.E. & Fanger M.W. 1984. Gamma interferon induces monocytoïd differentiation in the HL-60 cell line. *J Clin Invest*, **73**, 1072-1077.
4. Bennett S. & Breit S.N. 1994. Variables in the isolation and culture of human monocytes that are of particular relevance to studies of HIV. *J Leukoc Biol*, **56**, 236-240.
5. Cassol E., Alfano M., Biswas P. & Poli G. 2006. Monocyte-derived macrophages and myeloid cell lines as targets of HIV-1 replication and persistence. *J Leukoc Biol*, **80**, 1018-1030.
6. Goasguen J.E., Bennett J.M., Bain B.J., Vallespi T., Brunning R. & Mufti G.J. 2009. Morphological evaluation of monocytes and their precursors. *Haematologica*, **94**, 994-997.
7. Goud T.J., Schotte C. & van Furth R. 1975. Identification and characterization of the monoblast in monocuclear phagocyte colonies grown *in vitro*. *J Exp Med*, **142**, 1180-1199.

8. Guyre P.M., Morganelli P.M. & Miller R. 1983. Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. *J Clin Invest*, **72**, 393-397.
9. Hamilton T.A. & Adams D.O. 1987. Molecular mechanisms of signal transduction in macrophages. *Immunol Today*, **8**, 151-158.
10. Hibbs M.S., Hoidal J.R. & Kang A.H. 1987. Expression of metalloproteinase that degrades native type V collagen and denatured collagens by cultured human alveolar macrophages. *J Clin Invest*, **80**, 1644-1650.
11. Kadoi K. 2000. Establishment of a canine monocyte cell line. *New Microbiol*, **23**, 441-444.
12. Kadoi K. 2006. An establishment of a feline monocytic cell line. *New Microbiol*, **29**, 219-222.
13. Kadoi K. 2010. Establishment of two chicken embryonic cell lines in a newly developed nutrient medium. *New Microbiol*, **33**, 393-397.
14. Kadoi K., Tsukise A., Shiba H., Ikeda K., Seki T. & Ariga T. 2001. Establishment of swine monocyte cell line. *New Microbiol*, **24**, 243-247.
15. Karlsson K.R., Cowley S., Martinez F.O., Shaw M., Minger S.L. & James M. 2008. Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. *Exp Hematol*, **36**, 1167-1175.
16. Kuwana M., Okazaki Y., Kodama H., Izumi K., Yasuoka H., Ogawa Y., Kawakami Y. & Ikeda Y. 2003. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J Leukoc Biol*, **74**, 833-845.
17. Kuwana M., Okazaki Y., Kodama H., Satoh T., Kawakami Y. & Ikeda Y. 2006. Endothelial differentiation potential of human monocyte-derived multipotential cells. *Stem Cells*, **24**, 2733-2743.
18. Ross J.A. & Auger M.J. 2002. The biology of the macrophage. *In* The macrophage, Second Ed. (B. Burke & C.E. Lewis, eds). Oxford University Press, Oxford, 1-72.
19. Santini S.M., Lapenta C., Logozzi M., Parlato S., Spada M., Di Pucchio T. & Belardelli F. 2000. Type 1 interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity *in vitro* and in Hu-PBL-SCID mice. *J Exp Med*, **191**, 1777-1788.
20. Seta N. & Kuwana M. 2007. Human circulating monocytes as multipotential progenitors. *Keio J Med*, **56**, 41-47.
21. Seta N. & Kuwana M. 2010. Derivation of multipotent progenitors from human circulating CD14+ monocytes. *Exp Hematol*, **38**, 557-563.
22. Shiba H., Kadoi K. & Sato T. 2006. Monocytes of cat and pig secrete non-metalloproteases whose activities can be dramatically enhanced by calcium. *In* Proc. 1st Joint Meeting of European National Societies of Immunology & 16th European Congress of Immunology (ECI), 6-9 September, Paris. ECI Congress Administrative Secretariat, Paris, PA-3077, 462 pp..
23. Twardy D.J., Fujiwara H., Scillian J.J. & Ellner J.J. 1986. Concurrent enhancement of monocyte immunoregulatory properties and effector functions by recombinant interferon-gamma. *Cell Immunol*, **100**, 34-46.
24. Utsugi T. & Sone S. 1986. Comparative analysis of the priming effect of human interferon-gamma, -alpha, and -beta on synergism with muramyl dipeptide analog for anti-tumor expression of human blood monocytes. *J Immunol*, **136**, 1117-1122.
25. Watanabe H., Nakanishi I., Yamashita K., Hayaakawa T. & Okada Y. 1993. Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. *J Cell Sci*, **104**, 991-999.
26. Welgus H.G., Campbell E.J., Cury J.D., Eisen A.Z., Senior R.M., Wilhelm S.M. & Goldberg G.I. 1990. Neutral metalloproteinases produced by human mononuclear phagocytes. Enzyme profile, regulation, and expression during cellular development. *J Clin Invest*, **86**, 1496-1502.
27. Yam L.T., Li C.Y. & Crosby W.H. 1971. Cytochemical identification of monocytes and granulocytes. *Am J Clin Pathol*, **55**, 283-290.