

## Comparison of Thp-1 Macrophages Viability in Different Types of Culture Vessel

Zahidah Nasuha Mohd Yasin, Fatin Najiah Mohd Idrus, Get Bee Yvonne-Tee \*

Biomedicine Programme, School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian 16150, Malaysia

### Article history:

Submission August 2022

Revised January 2023

Accepted January 2023

\*Corresponding author:

E-mail: [yvonneteet@usm.my](mailto:yvonneteet@usm.my)

### ABSTRACT

The ox-LDL generated apoptotic bodies using THP-1 macrophage is a useful tool to study foam cell formation in atherosclerosis. However, the cells in the negative control group undergo apoptosis in the absence of ox-LDL. Therefore, the type of cell culture vessel was proposed to be the key factor contributing to cell apoptosis. The THP-1 cells were differentiated into M1 macrophages using 10 ng/μL PMA, 5 ng/μL LPS, and 20 ng/μL IFN-γ while 5 ng/μL PMA, 20 ng/μL IL-4 and 20 ng/μL IL-13 were used to differentiate THP-1 into M2 macrophages. Two types of cell culture vessels (6-well plate and T25 flask) were used to culture the macrophages. The cells were stained using Annexin V-FITC and Propidium Iodide before flow cytometry analysis. Interestingly, both M1 and M2 macrophages cultured in the T25 flask resulted in a significantly higher percentage of cell viability compared to macrophages cultured in 6-well plate [M1: 84.15% ± 4.39 vs 8.02% ± 1.55,  $p < 0.0001$ ; M2: 95.95% ± 1.74 vs 10.50% ± 0.05,  $p < 0.0001$ ]. In summary, the type of culture vessel is a vital factor in determining cell viability attributed to the surface area and cell seeding density in different types of vessels.

*Keywords:* Apoptosis, Culture vessel, Macrophage, THP-1, Viability

### Introduction

Efferocytosis is a highly regulated process which responsible for the clearance of daily-generated dying cells (i.e. apoptotic bodies) in the physiological state of the human body [1]. The engulfment of apoptotic bodies by professional efferocyte such as macrophages provides a protective mechanism from the detrimental effect of the apoptotic body build-up. The efferocytosis is also essential in inflammation resolution as the unengulfed apoptotic bodies can easily undergo secondary necrosis and release inflammatory contents that may subsequently cause inflammatory disorder [2, 3]. The significance of efferocytosis is highlighted in inflammatory disorders such as atherosclerosis [4, 5]. In atherosclerosis, the efferocytosis mechanism is hampered, accumulating apoptotic bodies in atherosclerotic plaque. The assemblage of apoptotic bodies in the plaque can trigger a plaque rupture, resulting in disastrous clinical outcomes such as myocardial infarction, ischemic stroke, and sudden cardiac death [6–8]. Therefore,

the study to elucidate the dysregulated efferocytosis mechanism is worthy of attention to discover potential novel biomarkers or therapeutic candidates for atherosclerosis.

During atherosclerosis, the gradual accumulation of oxidized low-density lipoprotein (ox-LDL) particles contributes to the formation of plaque in the artery [1, 4]. Macrophages are responsible for ox-LDL uptake and transforming them into foam cells. The lipid-engorged foam cells are susceptible to diverse cellular death pathways, including apoptosis which further turns them into apoptotic bodies [9, 10]. We are interested in generating *in vitro* apoptotic bodies model using macrophages derived from THP-1 cell line to mimic the pathological state of atherosclerosis.

THP-1 cell line is among the potential *in vitro* surrogate of primary macrophages because it has unlimited sources due to its infinite life span, possesses a homogenous background as they originated from a single source, and can eliminate the

### How to cite:

Yasin ZNM, Idrus FNM, Bee YTG (2023) Comparison of Thp-1 Macrophages Viability in Different Types of Culture Vessel. Journal of Tropical Life Science 13 (2): 359 – 368. doi: 10.11594/jtls.13.02.14.

invasive procedure of human blood withdrawal [11, 12]. The generation of apoptotic bodies in THP-1 macrophage induced by ox-LDL can serve as a substrate for efferocytosis. The apoptotic body formation can be detected using flow cytometry by assessing Annexin V FITC and Propidium Iodide (PI) staining on the cell. During apoptosis, phosphatidylserine is translocated to the external membrane, which serves as an apoptotic recognition molecule recognized by Annexin V FITC. The macrophage also can undergo another cellular death pathway, such as necrosis, in which they will release nucleus contents after their death which can be detected by DNA binding dye, PI. This allows the discrimination of viable cells (FITC-PI-), early apoptotic cells (FITC+PI-), late apoptotic cells (FITC-PI+) and necrotic cells (FITC+PI+), which can be detected by flow cytometry [13].

However, the common challenge in the apoptosis assay is that the negative control group cells undergo apoptosis. Generally, a negative control group is a group that receives no treatment and is expected to survive in the absence of the intervention [14]. Therefore, the validity and reliability of any intervention in the treatment group could be challenged and invalid if the cells in the negative control group undergo apoptosis. An expected occurrence happens, which is the THP-1 macrophage in the negative control group undergoes apoptosis without induction of ox-LDL. Until now, a limited study is available investigating the reason for cell lines, particularly THP-1 susceptibility to apoptosis in the absence of an apoptosis inducer. Due to the growth of adherent cells, including macrophages depending on the availability of surface area [15], we proposed that different types of cell surface area in culture vessels impact the viability of THP-1 macrophages. Two types of culture vessels which are the culture flask (T-25 flask) and well plate (6-well plate) were investigated for THP-1 culture and differentiation into M1 and M2 macrophages. The differences in the culture environment provided by the two culture vessels may lead to the disparity of viability percentage in macrophages.

The findings from this study can provide significant inputs for the optimization of apoptosis assay involving cell lines, particularly THP-1. Several other macrophage cell lines are available as an alternative for primary macrophages, such as RAW 264.7 and U937 [16], however, THP-1-

derived macrophages possess the closest characteristics as primary macrophages in terms of phagocytosis, pro-inflammatory and anti-inflammatory cytokines secretion as well as lipid uptake [17, 18]. The involvement of cells in a laboratory experiment is time-consuming as it requires the optimization of numerous factors. In this study, we evaluated the effect of two different culture conditions on the viability THP-1 after differentiation into M1 and M2 macrophages. We demonstrated that the condition of the culture vessel is critical for the viability of THP-1 macrophage.

## Material and Methods

### THP-1 culture

THP-1 cell line was cultured in RPMI-1640 media (Corning, USA) supplemented with 10% fetal bovine serum (Capricorn Scientific, USA), and 1% of penicillin/streptomycin (Corning, USA). The THP-1 culture was maintained at 5% CO<sub>2</sub>, 37°C and the media was replaced every two or three days a week.

### THP-1 macrophage differentiation

THP-1 cell line was differentiated into M1 macrophage using 10 ng/μL PMA (phorbol 12-myristate 13-acetate; Targetmol, USA), 5 ng/μL LPS (Lipopolysaccharides; Sigma, USA) and 20 ng/μL IFN-γ (Interferon-gamma; Stem Cell Technologies, Canada) while 5 ng/μL PMA, 20 ng/μL IL-4 (Interleukin-4; Peprotech, USA) and 20 ng/μL IL-13 (Interleukin-13; Peprotech, USA) were used to differentiate THP-1 into M2 macrophage. The full differentiation of M1 and M2 macrophages occurred in seven days. The media were changed every three days through day seven. The macrophages were differentiated into two types of cell culture vessels: a T-25 flask (SPL Life Sciences, Korea) and a 6-well plate (True Line, USA). The cell seeding number for THP-1 macrophage differentiation was  $1 \times 10^6$  and  $3 \times 10^5$  in T25-flask and 6-well plate, respectively. The differentiated M1 and M2 macrophages in a flask and well plate were harvested using cell detachment solution, Accumax (Thermo Fisher Scientific, USA) and cell scraper (NEST, China) before flow cytometry analysis.

### Characterization of M1 and M2 macrophages derived from THP-1

The phenotypic characteristic, and cell surface markers of M1 and M2 macrophages were verified

using flow cytometry analysis. The harvested macrophage was incubated with 2  $\mu\text{l}$  of CD86-FITC human clone FM95 (M1 surface marker) and 2  $\mu\text{l}$  of CD200R-PE human clone REA725 (M2 surface marker) (Miltenyi Biotec, German) in 100  $\mu\text{l}$  of MACS buffer for 10 minutes at 4°C in the dark. After the final washing step, the labeled macrophages were analyzed in 400  $\mu\text{l}$  of MACS buffer using a flow cytometry machine (BD FACSCanto).

### Apoptosis assay

The harvested macrophages were stained using FITC Annexin V Apoptosis Detection Kit with PI (Biolegend, California). The macrophages were incubated with 5  $\mu\text{l}$  of Annexin V and 3  $\mu\text{l}$  of PI in 400  $\mu\text{l}$  of binding buffer for 15 minutes at room temperature in the dark. The fluorescent signal of Annexin V and PI expressed by macrophages was evaluated using a flow cytometry machine (BD FACSCanto). Macrophages with both Annexin V and PI negative were considered healthy and viable, macrophages with PI negative and Annexin V positive were considered early apoptotic, macrophages with PI positive and Annexin V negative were considered late apoptotic and macrophages with both PI and Annexin V positive were considered necrotic.

### Statistical analysis

Quantitative data were managed using GraphPad Prism Version 8. The data were expressed as mean  $\pm$  SD. The independent t-test was employed to determine the significant difference in parameters between the two groups. The statistical test was considered significant when the p-value was  $< 0.05$ .

## Results and Discussion

### THP-1 differentiation into M1 and M2 macrophages

THP-1 is a monocytic cell line that has been extensively employed as *in vitro* cell model of primary macrophage. THP-1 was first discovered in a 1-year-old male patient with acute monocytic leukemia [19, 20]. The use of THP-1 is hugely beneficial in the immunological field as it can be used as a surrogate *in vitro* tool to study the monocyte/macrophage function [21–23]. THP-1 were selected as the primary tool in the present study due to the similitude of their function with primary macrophage in terms of surface markers

expression, phagocytosis capability, lipid uptake, and secretion of inflammatory cytokines [22, 24, 25]. The main advantages of employing THP-1 over primary macrophages are their endless sources owing to the eternal shelf life of cell line [12, 20]. Several cell lines are available as an alternative to primary macrophages such as U937 and HL-6p [12, 16], but THP-1 is the most commonly utilized as they shared the closest characteristic of primary macrophages, notably in the surface markers expression [24, 26].

THP-1 can differentiate into resting macrophages (M0) using PMA and polarized into M1 and M2 macrophages using LPS, IFN- $\gamma$ , IL-4 and IL-13. A 72-hour incubation in the presence of 5 and 10 ng/ $\mu\text{L}$  PMA to induce THP-1 cell line differentiation into resting macrophage followed by a 72-hour incubation with M1 stimulants (5 ng/ $\mu\text{L}$  LPS & 20 ng/ $\mu\text{L}$  IFN- $\gamma$ ) and M2 stimulants (20 ng/ $\mu\text{L}$  IL-4 & 20 ng/ $\mu\text{L}$  IL-13) to induce polarization into M1 and M2 macrophage was selected as differentiation protocol [24, 27, 19]. To allow full THP-1 differentiation into resting macrophages, 24 hours resting period (i.e. macrophage in media only) was implemented after PMA treatment prior to stimulation of M1 and M2 stimulants [24].

The macrophage surface markers expression, CD86 and CD200R were analyzed via flow cytometric analysis to verify the THP-1 monocyte differentiation into M1 and M2 macrophages. A significantly higher CD86 expression was observed in M1 macrophages compared to M2 macrophages attributed to the exposure of LPS and IFN- $\gamma$  [M1:  $2.71 \pm 0.69$  vs M2:  $1.07 \pm 0.06$ ,  $p = 0.014$ ] (Figure 1A). The findings generated from this study were consistent with previous studies indicating that M1 macrophages exhibited a higher CD86 signal than M2 macrophages [20, 28]. CD200R serve as M2 marker was induced in M2 macrophage using IL-4 and IL-13. According to Figure 1B, M2 macrophage significantly expressed higher CD200R than M1 macrophage [M1:  $1.01 \pm 0$  vs M2:  $1.25 \pm 0.02$ ,  $p < 0.0001$ ]. Purcu and colleagues also reported a higher CD200R expression in M2 macrophages compared to M1 macrophages after stimulation of IL-4 and IL-13 [29].

### Apoptotic bodies formation in the negative control group

Apoptosis is the most prominent event in atherosclerotic plaque and is a crucial contributing

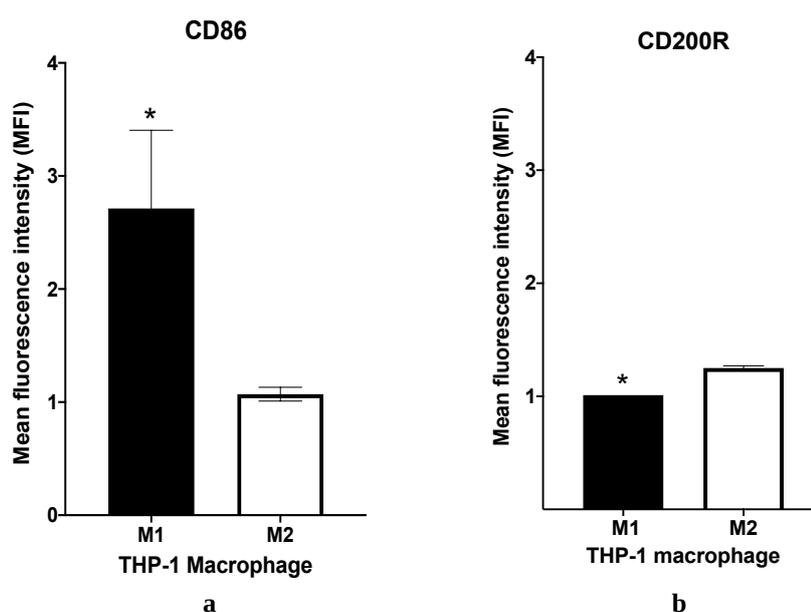


Figure 1. CD86 (a) and CD200R (b) expression in M1 and M2 macrophages. Cell surface markers of CD86 and CD200R were measured via flow cytometric analysis. The data represent the mean fluorescence intensity (MFI) of three biological replicates and the statistical differences between the two groups were tested by an independent t-test. As shown in Figure 1A and B, the mean expression of M1 marker, CD86 was significantly higher in M1 macrophage [M1:  $2.71 \pm 0.69$  vs M2:  $1.07 \pm 0.06$ ,  $p = 0.014$ ] and M2 marker, CD200R was significantly higher in M2 macrophage [M1:  $1.01 \pm 0$  vs M2:  $1.25 \pm 0.02$ ,  $p < 0.0001$ ].

factor to plaque vulnerability [10, 9]. The rupture of vulnerable plaque can progress to acute thrombosis, the terminal event that provokes acute myocardial infarction, sudden cardiac death and stroke [7, 30]. Macrophages lead to reducing the atherosclerotic plaque burden by engulfing the accumulated ox-LDL and transforming them into foam cells [31]. The lipid overload in the macrophage foam cell can induce apoptosis of the cell by activating the apoptotic pathway. The lipid particles of ox-LDL can induce apoptosis through the caspase cascade, such as caspase 9, caspase 3, and caspase 7 [9, 32]. Interestingly, macrophages also can serve as efferocyte to clear the apoptotic bodies, a process known as efferocytosis. In atherosclerosis conditions, the efferocytosis is dysregulated and leads to the accumulation of apoptotic bodies [5]. Therefore, to better understand how efferocytosis is dysregulated in atherosclerosis, investigating apoptotic body formation induced by ox-LDL can provide useful and practical insight. However, the problem in the present study is both M1 and M2 macrophage-derived from THP-1 in the negative control group were susceptible to apoptosis without the presence of ox-LDL. The apoptotic body formation was assessed using FITC Annexin V Apoptosis Detection Kit with PI.

The percentage of viable cells in M1 and M2 macrophages was 9.70 % and 10.02 %, respectively (Figure 2A and B).

Several variables may induce low cell viability in the negative control group. In the cell culture environment, macrophages are highly adherent cell types and improper cell detachment methods can interfere with the cell viability [33]. Tsuji et al. (2017) reported that different types of cell detachment reagents resulted in different outcomes of cell viability as well as surface marker expression. They suggested that TrypLE solution is superior to other cell dissociation reagents as cell viability was not significantly altered and the treatment did not affect the expression of any cell surface marker after 30 minutes of the treatment [34]. This study employed the Accumax solution as a detachment reagent to detach macrophages from the culture vessel. We attempted changing to TrypLE as a detachment reagent for macrophages; however, the outcomes of cell viability percentage were similar to the macrophages that were treated with Accumax, [TrypLE:  $7.23 \% \pm 1.27$  vs Accumax:  $6.50 \% \pm 0.81$ ] (Data not shown).

Moreover, in the present study, a cell scraper usage was established for the macrophage detachment process to maximize the yield of the har-

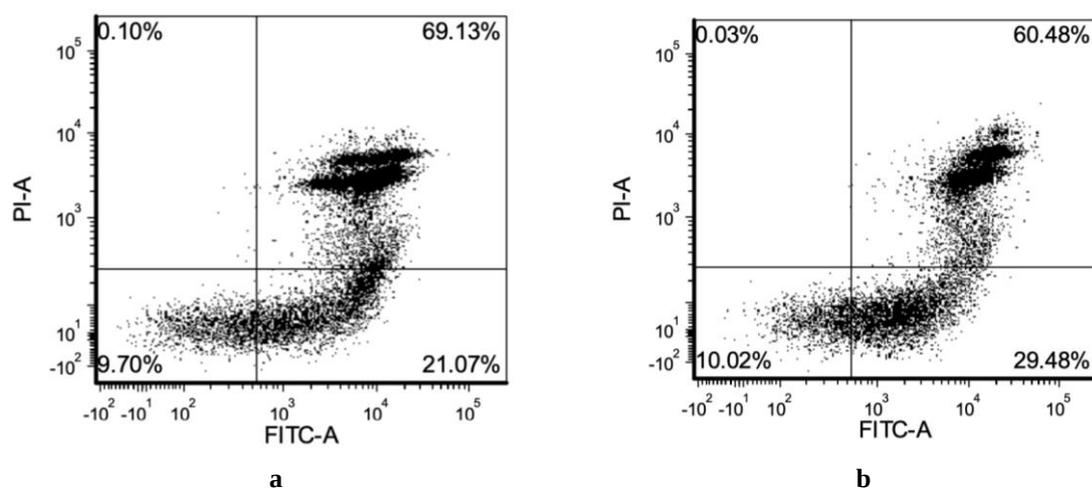


Figure 2. Apoptotic bodies formation in negative control group: (A) apoptotic bodies formation in negative control of M1 macrophage, and (B) apoptotic bodies formation in negative control of M2 macrophage. Macrophage with both Annexin V and PI negative were considered healthy and viable, macrophage with PI negative and Annexin V positive cells were considered early apoptotic, macrophage with PI positive and Annexin V negative cells were considered late apoptotic and macrophage with both PI and Annexin V positive were considered necrotic. M1 and M2 macrophages form apoptotic bodies in the absence of apoptosis inducer, ox-LDL. The percentage of viable cells in M1 and M2 macrophages were 9.70 % and 10.02 %, respectively.

vested cells. According to previous studies, excessive mechanical handling of the adherent cell using a cell scraper can affect the cellular membrane integrity in a human cell line, exposing phosphatidylserine and nucleus contents that Annexin V and PI can positively detect. In addition to that, a prior study revealed that the detachment method using a cell scraper is considered an ineffective way of harvesting cell lines as their usage significantly reduced cell viability, ranging from 34.6% to 87.18 % [35, 36]. We attempted the elimination of the cell scraper application in order to increase the macrophage viability, but the percentage of viable cells remained low, [Harvested macrophage with scraper:  $8.29 \% \pm 1.09$  vs harvested macrophage without scraper:  $9.22 \% \pm 0.91$ ] (Data not shown). Thus, it demonstrated that this study's apoptosis of M1 and M2 macrophages was less likely attributed to the common factors indicated above.

#### **Viability of THP-1 macrophage in two different culture vessel conditions**

There was increasing evidence proving that the cell detachment technique can affect cell viability in the cell line [33–36]. To date, no research has explored how different conditions in culture vessels can affect cell viability. Therefore, a different type of culture vessel condition was

proposed to be a key apoptosis factor that may have an impact on the viability of THP-1 macrophages as the growth of adherent cells is limited by available surface area. The T-25 flask and 6-well plate were investigated to determine the effect of different cell culture vessel conditions on cell viability.

A good cell viability percentage must be between 80 to 90 %. In line with our prediction, the dot plot shown in Figure 3A, 80.95 % of M1 macrophages survive in the T-25 flask as opposed to only 9.70 % of M1 macrophages viable in the 6-well plate (Figure 3B). The dot plot shown in Figures 4A and B also revealed higher viability obtained by M2 macrophages that were cultured in the T-25 flask (M2 in T-25 flask: 96.38% vs M2 in 6-well plate: 10.02 %). The data were analyzed using an independent t-test. A significant increase of cell viability percentages were observed in M1 macrophage (Figure 3 C) that cultured in T-25 flask compared to 6-well plate, [M1:  $84.15 \% \pm 4.39$  vs  $8.02 \% \pm 1.55$ ,  $p < 0.0001$ ]. A similar pattern was observed in M2 macrophage (Figure 4 C) in which the macrophage viability percentages significantly increased after being cultured in T-25 flask compared to with 6-well plate, [M2:  $95.95 \% \pm 1.74$  vs  $10.50 \% \pm 0.05$ ,  $p < 0.0001$ ]. In general, cell attachment to the substrate, a culture vessel, is an early stage for the adherent cell to

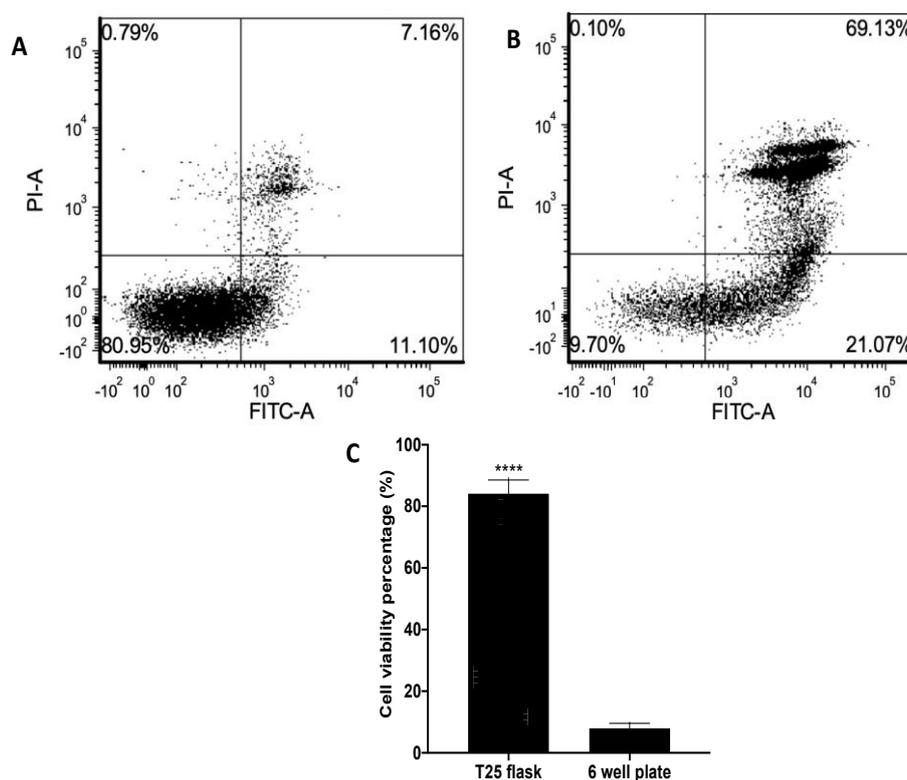


Figure 3. Comparison of viable percentage of M1 macrophage in T-25 flask versus 6-well plate: (A) the dot plot shows the apoptosis and viability of M1 macrophage cultured in T-25 flask, (B) The dot plot shows the apoptosis and viability of M1 macrophage cultured in 6-well plate, and (C) the bar graph shows the difference of viable M1 macrophage percentage of 3 biological replicates in T-25 flask and 6-well plate and the statistical differences between two groups were tested by an independent t-test. A significant difference in viable cell percentage was observed in M1 macrophage cultured in T-25 flask versus 6-well plate,  $84.15\% \pm 4.39$  vs  $8.02\% \pm 1.55$ ; ( $p < 0.0001$ ), respectively.

proliferate and differentiate [37]. THP-1 is a monocytic cell that manifests as a suspension cell in a culture environment. Upon exposure to specific stimulants, they will differentiate into adherent cell type macrophage by adhering to the culture vessels. The findings from this study suggested that different conditions of culture vessels can affect the viability of THP-1 macrophages. The T-25 flask provides a greater surface area than the 6-well plate for THP-1 differentiation into macrophages, which increases cell viability percentage. The idea was supported by a previous study which highlighted that increased culture surface area is able to maximize the growth of the cells [38]. Besides, the adherent cell, including macrophages, also requires an attachment to the substrate in the culture environment for their survival [39]. The 6-well plate provides a smaller surface area compared to the T-25 flask, which may cause insufficiency of macrophage attachment that eventually promotes apoptosis. Moreover, Ponath and Kaina revealed that monocytes are more susceptible to

death than macrophages due to superior survival ability [40]. Therefore, the difference in surface area of different culture vessel types can promote disparity and profoundly affect the study outcomes.

In addition to that, the T-25 flask offers a higher cell seeding number for cell culture compared to the 6-well plate. The results demonstrated that cell seeding numbers could affect cell viability because M1 and M2 macrophages could survive well in the T25 flask. It is supported by the study of Zhou and his colleagues, which reported that cell viability greatly increases by increasing cell seeding number in human mesenchymal stem cells [41]. However, these findings were contradicted by the study of adipose tissue-derived stem cells in which increasing cell seeding number leads to a reduction of cell viability [42]. The difference among the studies may be due to different cell types that behave differently in certain *in vitro* culture conditions.

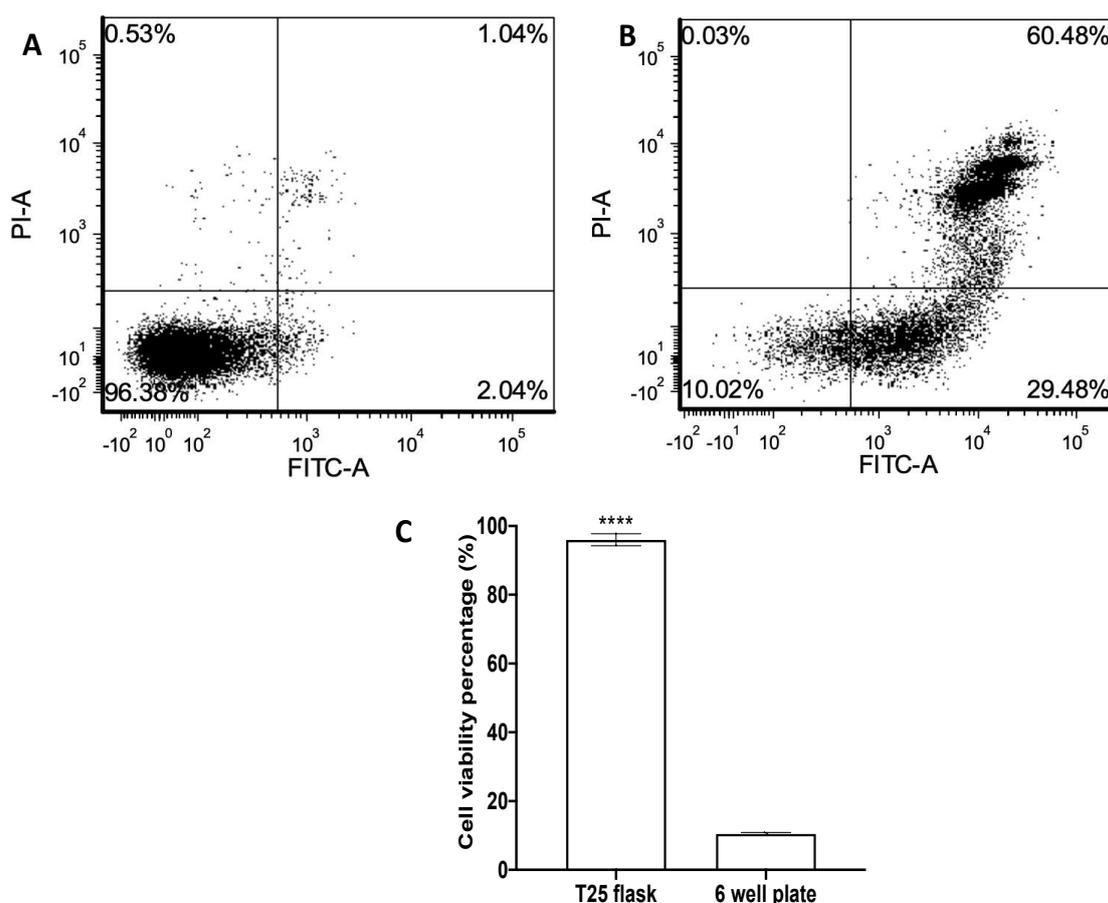


Figure 4. Comparison of viable percentage of M2 macrophage in T-25 flask versus 6-well plate: (A) The dot plot shows the apoptosis and viability of M2 macrophage cultured in T-25 flask, (B) The dot plot shows the apoptosis and viability of M2 macrophage cultured in 6-well plate, and (C) The bar graph shows the difference in viable M2 macrophage percentage of 3 biological replicates in T-25 flask and 6-well plate and the statistical differences between two groups were tested by independent t-test. A significant difference in viable cell percentage was observed in M2 macrophage cultured in T-25 flask versus 6-well plate,  $95.95 \pm 1.74$  vs  $10.50 \pm 0.05$ ; ( $p < 0.0001$ ), respectively.

Furthermore, a different culture media volume in different culture vessels can have an impact on the cellular interaction in cell culture as they provide different nutrient concentrations and allow different cellular waste exchanges [43]. Insufficient proper nutrients can affect cell viability in the *in vitro* culture experiments [44]. Finally, yet importantly, using a flask instead of a well plate benefits the routine of cell culture maintenance as they provide better protection from contamination attributed to a secure screw cap.

## Conclusion

Optimization of experimental conditions is critical in ensuring robust experimental reproducibility, validity, and reliability. The obtained findings in this study can provide useful insight for the optimization of cell culture assay. The present

study highlighted that different types of culture vessels can significantly affect the growth and viability of the cell. Hence, the type of culture vessel is a vital factor in determining cell viability attributed to the difference in surface area and cell seeding number. Therefore, optimizing the basal condition of cell culture assay is crucial for the downstream experiment's reliability and reproducibility.

## Acknowledgment

This work was supported by the Research University Grant, RUI: 1001/PPSK/8012272 and the Ministry of Higher Education Malaysia for Fundamental Research Grant Scheme with Project Code: FRGS/1//2018/SKK06/USM/02/2. The abstract of this work was presented during the International

Conference of Bioscience and Medical Engineering (ICBME 2022).

## References

- Kojima Y, Weissman IL, Leeper NJ (2017) The Role of Efferocytosis in Atherosclerosis. *Circulation* 135 (5): 476. doi: 10.1161/circulationaha.116.025684.
- Kourtzelis I, Hajishengallis G, Chavakis T (2020) Phagocytosis of Apoptotic Cells in Resolution of Inflammation. *Frontiers in Immunology* 11 553. doi: 10.3389/fimmu.2020.00553/bibtext.
- Park SY, Kim IS (2017) Engulfment signals and the phagocytic machinery for apoptotic cell clearance. *Experimental & Molecular Medicine* 49 (5): e331. doi: 10.1038/emmm.2017.52.
- Silvestre-Roig C, De Winther MP, Weber C et al. (2014) Atherosclerotic plaque destabilization: Mechanisms, models, and therapeutic strategies. *Circulation Research* 114 (1): 214–226. doi: 10.1161/circresaha.114.302355.
- Doran AC, Yurdagul A, Tabas I (2020) Efferocytosis in health and disease. *Nature Reviews Immunology* 20 (4): 254–267. doi: 10.1038/s41577-019-0240-6.
- Gerhardt T, Haghikia A, Stapmanns P, Leistner DM (2022) Immune Mechanisms of Plaque Instability. *Frontiers in Cardiovascular Medicine* 0 2092. doi: 10.3389/fcvm.2021.797046.
- Stefanadis C, Antoniou CK, Tsiachris D, Pietri P (2017) Coronary Atherosclerotic Vulnerable Plaque: Current Perspectives. *J Am Heart Assoc.* doi: 10.1161/jaha.117.005543
- Soehnlein O, Libby P (2021) Targeting inflammation in atherosclerosis — from experimental insights to the clinic. *Nature Reviews Drug Discovery* 20:8 20 (8): 589–610. doi: 10.1038/s41573-021-00198-1.
- Gui Y, Zheng H, Cao RY (2022) Foam Cells in Atherosclerosis: Novel Insights Into Its Origins, Consequences, and Molecular Mechanisms. *Frontiers in Cardiovascular Medicine* 0 842. doi: 10.3389/fcvm.2022.845942.
- Moore KJ, Sheedy FJ, Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. *Nature reviews Immunology* 13 (10): 709. doi: 10.1038/NRI3520.
- Forrester MA, Wassall HJ, Hall LS et al. (2018) Similarities and differences in surface receptor expression by THP-1 monocytes and differentiated macrophages polarized using seven different conditioning regimens. *Cellular Immunology* 332 58–76. doi: <https://doi.org/10.1016/j.cellimm.2018.07.008>.
- Qin Z (2012) The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis* 221 (1): 2–11. doi: <https://doi.org/10.1016/j.atherosclerosis.2011.09.003>.
- Kumar R, Saneja A, Panda AK (2021) An Annexin V-FITC—Propidium Iodide-Based Method for Detecting Apoptosis in a Non-Small Cell Lung Cancer Cell Line. *Methods in Molecular Biology* 2279 213–223. doi: 10.1007/978-1-0716-1278-1\_17/cover.
- Moser P (2020) Out of Control? Managing Baseline Variability in Experimental Studies with Control Groups. *Handbook of Experimental Pharmacology* 257 101–117. doi: 10.1007/164\_2019\_280/figures/3.
- Huang Z, Xie L, Li H et al. (2019) Insight into interleukin-37: The potential therapeutic target in allergic diseases. *Cytokine & Growth Factor Reviews* 49 32–41. doi: <https://doi.org/10.1016/j.cytogfr.2019.10.003>.
- Aldo PB, Craveiro V, Guller S, Mor G (2013) Effect of culture conditions on the phenotype of THP-1 monocyte cell line. *American journal of reproductive immunology* (New York, NY: 1989) 70 (1): 80. doi: 10.1111/aji.12129.
- Pauls SD, Rodway LA, Winter T et al. (2018) Anti-inflammatory effects of  $\alpha$ -linolenic acid in M1-like macrophages are associated with enhanced production of oxylipins from  $\alpha$ -linolenic and linoleic acid. *The Journal of Nutritional Biochemistry* 57 121–129. doi: <https://doi.org/10.1016/j.jnutbio.2018.03.020>.
- Shiratori H, Feinweber C, Luckhardt S et al. (2017) THP-1 and human peripheral blood mononuclear cell-derived macrophages differ in their capacity to polarize in vitro. *Molecular Immunology* 88 58–68. doi: <https://doi.org/10.1016/j.molimm.2017.05.027>.
- Genin M, Clement F, Fattaccioli A et al. (2015) M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer* 15 (1): 1–14. doi: 10.1186/s12885-015-1546-9.
- Forrester MA, Wassall HJ, Hall LS et al. (2018) Similarities and differences in surface receptor expression by THP-1 monocytes and differentiated macrophages polarized using seven different conditioning regimens. *Cellular Immunology* 332 58–76. doi: 10.1016/j.cellimm.2018.07.008.
- Alhamdi JR, Peng T, Al-Naggar IM et al. (2019) Controlled M1-to-M2 transition of aged macrophages by calcium phosphate coatings. *Biomaterials* 196 90–99. doi: <https://doi.org/10.1016/j.biomaterials.2018.07.012>.
- Zhang H, Wu X, Wang G et al. (2018) Macrophage polarization, inflammatory signaling, and NF- $\kappa$ B activation in response to chemically modified titanium surfaces. *Colloids and Surfaces B: Biointerfaces* 166 269–276. doi: <https://doi.org/10.1016/j.colsurfb.2018.03.029>.
- Liao C-W, Chou C-H, Wu X-M et al. (2020) Interleukin-6 plays a critical role in aldosterone-induced macrophage recruitment and infiltration in the myocardium. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1866 (3): 165627. doi: <https://doi.org/10.1016/j.bbadis.2019.165627>.
- Daigneault M, Preston JA, Marriott HM et al. (2010) The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One.* doi: 10.1371/journal.pone.0008668
- Huleihel L, Dziki JL, Bartolacci JG et al. (2017) Macrophage phenotype in response to ECM bioscaffolds. *Seminars in Immunology* 29 2–13. doi: <https://doi.org/10.1016/j.smim.2017.04.004>.
- Tedesco S, De Majo F, Kim J et al. (2018) Convenience versus biological significance: Are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying in vitro polarization? *Frontiers in Pharmacology* 9 (FEB): 1–13. doi: 10.3389/fphar.2018.00071.
- Schlieffsteiner C, Ibesich S, Wadsack C (2020) Placental hofbauer cell polarization resists inflammatory cues in vitro. *Int J Mol Sci.* doi: 10.3390/ijms21030736
- Li P, Hao Z, Wu J et al. (2021) Comparative Proteomic

- Analysis of Polarized Human THP-1 and Mouse RAW264.7 Macrophages. *Front Immunol.* doi: 10.3389/fimmu.2021.700009/pdf
29. Purcu DU, Korkmaz A, Gunalp S et al. (2022) Effect of stimulation time on the expression of human macrophage polarization markers. *PLoS One.* doi: 10.1371/journal.pone.0265196
30. Bentzon JF, Otsuka F, Virmani R, Falk E (2014) Mechanisms of Plaque Formation and Rupture. *Circulation Research* 114 (12): 1852–1866. doi: 10.1161/circresaha.114.302721.
31. Brophy ML, Dong Y, Wu H et al. (2017) Eating the Dead to Keep Atherosclerosis at Bay. *Frontiers in Cardiovascular Medicine* 4 2. doi: 10.3389/fcvm.2017.00002/bibtex.
32. Ramji DP, Davies TS (2015) Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. *Cytokine and Growth Factor Reviews* 26 (6): 673–685. doi: 10.1016/j.cytogfr.2015.04.003.
33. Lai TY, Cao J, Ou-Yang P et al. (2022) Different methods of detaching adherent cells and their effects on the cell surface expression of Fas receptor and Fas ligand. *Scientific Reports* 2022 12:1 12 (1): 1–8. doi: 10.1038/s41598-022-09605-y.
34. Tsuji K, Ojima M, Otabe K et al. (2017) Effects of Different Cell-Detaching Methods on the Viability and Cell Surface Antigen Expression of Synovial Mesenchymal Stem Cells. *Cell Transplantation* 26 (6): 1089. doi: 10.3727/096368917X694831.
35. Yan G, Efferth T (2018) Cell Harvesting Methods Affect Cellular Integrity of Adherent Cells During Apoptosis Detection. *Anticancer research* 38 (12): 6669–6672. doi: 10.21873/anticancer.13034.
36. Nowak-Terpiłowska A, Ślędziński P, Zeyland J (2021) Impact of cell harvesting methods on detection of cell surface proteins and apoptotic markers. *Brazilian Journal of Medical and Biological Research* 54 (2): 1–7. doi: 10.1590/1414-431x202010197.
37. Hacking SA, Khademhosseini A (2013) Cells and Surfaces in vitro. *Biomaterials Science: An Introduction to Materials: Third Edition* 408–427. doi: 10.1016/b978-0-08-087780-8.00037-1.
38. Bellani CF, Ajeian J, Duffy L et al. (2020) Scale-Up Technologies for the Manufacture of Adherent Cells. *Frontiers in Nutrition* 7 178. doi: 10.3389/fnut.2020.575146/bibtex.
39. Krutty JD, Schmitt SK, Gopalan P, Murphy WL (2016) Surface Functionalization and Dynamics of Polymeric Cell Culture Substrates. *Current opinion in biotechnology* 40 164. doi: 10.1016/J.copbio.2016.05.006.
40. Ponath V, Kaina B (2017) Death of Monocytes through Oxidative Burst of Macrophages and Neutrophils: Killing in Trans. *PLoS One.* doi: 10.1371/journal.pone.0170347
41. Zhou H, Weir MD, Xu HHK (2011) Effect of Cell Seeding Density on Proliferation and Osteodifferentiation of Umbilical Cord Stem Cells on Calcium Phosphate Cement-Fiber Scaffold. *Tissue Engineering Part A* 17 (21–22): 2603. doi: 10.1089/ten.tea.2011.0048.
42. Sukho P, Kirpensteijn J, Hesselink JW et al. (2017) Effect of Cell Seeding Density and Inflammatory Cytokines on Adipose Tissue-Derived Stem Cells: an in Vitro Study. *Stem Cell Reviews and Reports* 13 (2): 267–277. doi: 10.1007/S12015-017-9719-3/figures/7.
43. Vis MAM, Ito K, Hofmann S (2020) Impact of Culture Medium on Cellular Interactions in in vitro Co-culture Systems. *Front Bioeng Biotechnol.* doi: 10.3389/fbioe.2020.00911/pdf
44. Arigony ALV, De Oliveira IM, Machado M et al. (2013) The influence of micronutrients in cell culture: a reflection on viability and genomic stability. *Biomed Res Int.* doi: 10.1155/2013/597282.

This page is intentionally left blank.