Determining the Effect of Cell Culture Methods on the Polarization and Phenotype of Macrophages

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Introduction

Macrophages are a common type of immune cell that play a significant role in the regulation of inflammation in the body (Ross et al., 2021). Macrophages are generally divided into two simplified phenotype groups, called M1 and M2, based on the way they promote or suppress inflammation. Additionally, macrophages can be polarized to either phenotype and can alter their phenotype based on their environmental signals, a phenomenon known as plasticity (Wynn et al., 2013). Macrophages polarized to the M1 phenotype are pro-inflammatory and contribute to the destruction of cells and limiting cell proliferation through the expression of IL-6 and tumor necrosis factor. On the other hand, macrophages with the M2 phenotype are anti-inflammatory and support cell and tissue growth through the expression of IL-10 and TGF-β (Yunna et al., 2020). In addition to these classical definitions, there is a cancer-specific tumor-associated macrophage (TAM) phenotype classification found in the tumor microenvironment (TME). The TAM phenotype is more similar to the M2 anti-inflammatory phenotype, where the macrophage, residing in a cancer tumor microenvironment, works to suppress the immune system, signal angiogenesis, and promote tumor growth (Mantovani et al., 2022). The main differences between the TAM and the M2 macrophage are that TAMs are mostly found within the TME and are more plastic than a typical M2 macrophage (Lee et al., 2019).

Due to their phenotypic plasticity, macrophages have the potential to be modulated, making them an attractive subject of cancer immunotherapy treatments (Mantovani et al., 2022). Therefore, cancer researchers, particularly those studying the role of macrophages in the tumor microenvironment, must be aware of the polarization of the macrophages they are studying to obtain consistent and meaningful results. This is important because different cell culture methods have been known to have significant impacts on macrophage phenotype polarization (Chen et al., 2015).

In this study, we aim to examine the influence of common cell culture variations on macrophage polarization by using flow cytometry to measure the expression of established surface markers. Based on established protocols, we expect that a peripheral blood mononuclear cell (PBMC) culture will reach complete differentiation into macrophages after 7 days of incubation with macrophage colony-stimulating factor (M-CSF). Additionally, it is expected that cell passaging with EDTA will cause the macrophages to differentiate into a predominantly M1 phenotype (Chen et al., 2015).

Methods

Differentiating PBMCs into macrophages

5 wells of human-derived PBMCs were seeded at 1.5e6 cells/well in a low-adhesion 6 well plate. Cells were cultured with 3 mL RPMI 1640 medium per well, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. M-CSF was added to the wells at a concentration of 60 ng/mL at seeding, then replenished with 120 ng/mL of M-CSF in 3 mL of FBS and pen/strep supplemented RPMI after 5 days. Cells were collected, fixed with 4% paraformaldehyde, and permeabilized with 90% methanol at 4, 5, 6, 7, and 9 days. The 5-day group was collected 1 h after the M-CSF was replenished. The permeabilized cells were stained for

surface marker CD68 and analyzed with flow cytometry using BD C6 Plus Flow Cytometer. Statistical analysis was performed using FlowJo software package.

Determining the effect of passaging on macrophage phenotype

PBMCs were seeded and cultured as described in the previous experiment. The passage group (n=3) was passaged after 5 days by 30 minutes of incubation 5mM EDTA in 1X phosphate-buffered saline (PBS). After 7 days of incubation in culture, the cells from both the passage group and the control group (n=3) were lifted, fixed, and permeabilized. The cells were divided into three groups for staining: a no stain control, a group stained for M1 surface markers, and a group stained for M2 surface markers. The M1 group was stained for CD68, CD38, and CD80, and the M2 group was stained for CD68, CD163, and CD206. The samples were then analyzed using flow cytometry. One of the passage groups was removed from analysis due to an insufficient number of cells for flow cytometry. Statistical analysis was performed using FlowJo software package.

Results

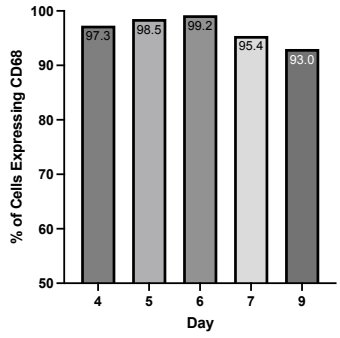

Figure 1. Quantitative analysis of the percentage of CD68⁺ cells in culture across the differentiation period (n=1).

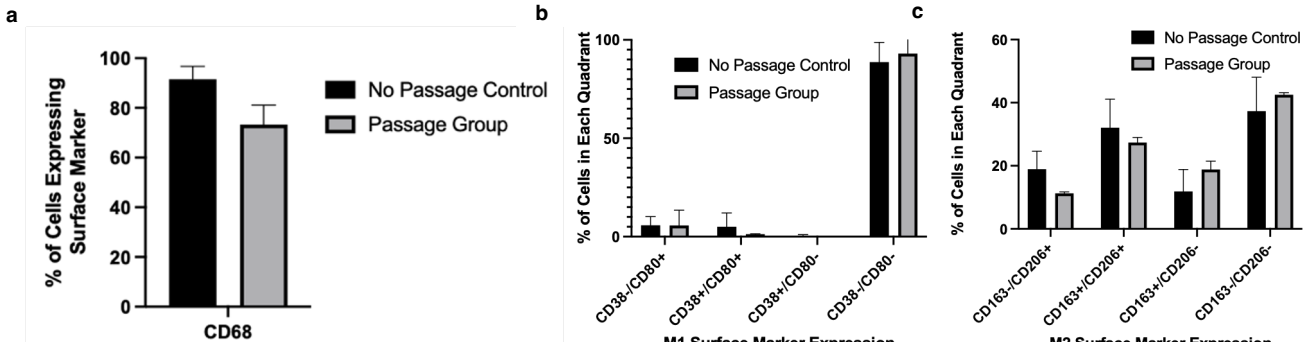

Figure 2. (a) Quantitative analysis of the percentage of CD68⁺ cells in culture. (b) Percentage of cells in quadrant gate quadrants for M1 surface markers, where Q1 contains CD38-/CD80+ cells, Q2 contains CD38+/CD80+ cells, Q3 contains CD38+/CD80- cells, and Q4 contains CD38-/CD80- cells. (c) Percentage of cells in quadrant gate quadrants for M2 surface markers CD163 and CD 206, where Q1 contains CD163-/CD206+ cells, Q2 contains CD163+/CD206+ cells, Q3 contains CD163+/CD206- cells, and Q4 contains CD163-/CD206- cells.

Discussion

This study revealed some insightful details into the effects of macrophage cell culture methodologies on in vitro macrophage behavior. First, it can be shown that the majority of the PBMCs have differentiated into $CD68^+$ macrophages after 5 days of exposure to M-CSF, with the percentage peaking at 99.2% after 6 days (Figure 1). This shows that PBMCs should be sufficiently differentiated into macrophages by day 6 of culture with M-CSF. This will help inform macrophage researchers' differentiation protocols and help them to develop more efficient protocols, which can expedite the experimentation process.

Second, there was a marked decrease in the percentage of cells expressing CD68, the pan-macrophage surface marker, in the passage group (Figure 2a). This could indicate that passaging disrupts the differentiation process from monocyte to macrophage. However, we noticed almost complete differentiation by day 5, the day on which the cells were passaged, in the time experiment (Figure 1), which suggests either that the differentiated macrophages are more adherent than the undifferentiated PMBCs or that passaging reduces the expression of CD68 through dedifferentiation. The former explanation is more likely and is consistent with the decrease in percentage of the M1 macrophages in the passage group (Figure 2b)., which is likely due to their strong adherence (Nadeem et al., 2024).

Furthermore, it can be seen that, although cell passaging produces no statistically significant difference in macrophage polarization, there are discernable differences in the expression of select surface markers. Primarily, passaging decreases the percentage of CD38+/CD80+ cells (Figure 2b), or strongly polarized M1 macrophages, in culture. This can possibly be attributed to the M1 phenotype's relatively resilient adhesion properties compared to the M2 phenotype (Nadeem et al., 2024), which would prevent the polarized M1 cells from

detaching during passaging and thus remove the cells from culture. Additionally, passaging drives more cells towards expressing CD163 while decreasing the percentage of cells expressing CD206 (Figure 2c). This is a logical result since CD163 is thought to play a key role in the wound-healing process (Schaer et al., 2006). However, there is no clear explanation for the lower expression of CD206 in the passage group.

In general, it can be seen that cell passaging results in a slightly higher percentage of unpolarized macrophages than the non-passaging protocol. Largely, however, both the passaging and non-passaging macrophage culture protocols resulted in a mixed group of macrophage phenotypes, with no strong polarizations towards either the M1 or M2 phenotypes. This result is desirable for generic macrophage research since it represents a more natural array of polarized macrophages. However, for phenotype-specific research or research seeking to replicate the TME, a different differentiation protocol should be utilized considering the significant population of unpolarized macrophages.

Overall, this work could benefit from future studies that measure macrophage polarization before and after passaging to understand the effect of phenotype adhesion properties on the results of this study. Additionally, it would be beneficial to understand the effects of different cell culture media and cancer cell co-culturing on macrophage phenotype polarization *in vitro*. Understanding how macrophages respond to cancer cells in co-culture could give greater insight to how cancerous cells direct immune interactions and create the macrophage niche of the TME. Moreover, more thorough studies with higher sample sizes could reduce the variation that limited the analysis of this study.

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