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Polarizing macrophages derived from human THP-1 cells in vitro: methods and protocols

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Macrophages derived from the human THP-1 cell line have been widely used as substitutes for primary macrophages in various macrophage-related studies. However, difficulties still exist in establishing THP-1 macrophage models. This research presents techniques for generating different phenotypes of activated macrophages derived from THP-1 cells by introducing specific stimuli and provides some potential markers to confirm each type of activated macrophage. It is hoped to provide novel and useful methods for scientific research and to help researchers explore this field more intuitively and effectively.

Macrophages play a crucial role as essential effector cells within the innate immune system during tissue homeostasis, injury, and repair processes (Bao et al., 2024). Typically, macrophages can be classified as classically activated M1 macrophages and alternatively activated M2 macrophages. Additionally, M2 macrophages are further subdivided into M2a, M2b, M2c, and M2d subtypes based on the applied stimulus and microenvironment (Huang et al., 2018b; Marrufo and Flores-Mireles, 2024). In current research, primary macrophages are considered ideal in vitro cell models as they retain numerous important markers and functions in vivo. Nevertheless, primary macrophages also possess disadvantages, including diverse genetic backgrounds, significant individual differences, and limited availability. Consequently, cell line-derived macrophages are frequently employed as substitutes. THP-1 is a human monocytic leukemia cell line, and the macrophages derived from it closely mimic the morphological and functional characteristics of primary macrophages as well as their differentiation markers. This cell line has been extensively utilized to investigate the functions, mechanisms, and signaling pathways related to macrophages while maintaining a consistent genetic background with limited variability (Chanput et al., 2014). Furthermore, THP-1 macrophages have been used in research focused on identifying macrophage markers (Huang et al., 2018a).

Although there is substantial literature on different methods for creating macrophage polarization models, many laboratories still encounter difficulties in successfully constructing them. The challenges in constructing polarization models primarily stem from the variability of external stimuli, the lack of specific and reliable markers, and the technical limitations of cell culture and manipulation. These factors jointly contribute to the obstacles faced by many laboratories, highlighting the need for further research and optimization to enhance the accuracy and reproducibility of macrophage polarization studies. Accordingly, this research outlines techniques for producing various phenotypes of activated macrophages sourced from human THP-1 cells through the

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introduction of specific stimuli. The experimental method is based on previous reports and the experience of our research group (Huang, et al., 2018b; Li et al., 2021; Li et al., 2022). We also provide a reference of the unique markers employed to confirm each type of activated macrophage.

Method for inducing differentiation of THP-1 cells into M0 macrophages.

1. THP-1 monocytes were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin in an incubator with 5% CO2 at 37 ℃. Before experimental use, cells were verified as free of mycoplasma contamination, and microscopic images demonstrated that the cells were viable and intact.
2. The THP-1 cells were treated with 10–100 ng/mL phorbol-12-myristate-13-acetate (PMA) for 24–48 h to differentiate into M0 macrophages (see Note 1).
3. Adherent M0 macrophages were round, small, and stereoscopic, with a visible halo. If the adherent cells showed heterogeneous morphology, including round and spindle-shaped cells, this indicated that the M0 macrophages had started to differentiate.
4. Throughout the following procedures involving macrophage polarization, a common preparatory step was to wash the macrophages and replace the medium with fresh culture medium to ensure optimal conditions for subsequent stimulations (see Note 2).

Procedure for polarizing M0 macrophages into classic M1 macrophages.

1. The M0 macrophages were stimulated with 50 ng/mL recombinant human interferon-γ (IFN-γ) and 15 ng/mL lipopolysaccharide (LPS) for 48 h to obtain the M1 phenotype, which was characterized by a spindle shape with increased pseudopodia.
2. The IFN-γ and LPS doses could be adjusted appropriately according to the actual situation. In this step, high concentrations of IFN-γ and LPS were avoided to prevent oxidative stress damage and excessive inflammation.

Detailed steps for polarizing M0 macrophages into alternative M2 macrophages.

1. The M0 macrophages were stimulated with 25 ng/mL recombinant human interleukin-4 (IL-4) and 25 ng/mL recombinant human interleukin-13 (IL-13) for 72 h to achieve the M2 phenotype. The filopodia of M2 macrophages were less densely distributed and appeared flattened and elongated (Lepur et al., 2012).
2. Similarly, the IL-4 and IL-13 doses could be adjusted as needed. It should be noted that macrophages require a certain amount of time to respond to interleukins, and a too-short stimulation time is insufficient to induce the polarization of M2 macrophages.

Specific method for polarizing M0 macrophages into M2a macrophages.

1. The M0 macrophages were stimulated with IL-4 (20 ng/mL) for 24–48 h to M2a phenotype.
2. This step is optional. In fact, treatment with IL-4 alone is sufficient to generate the M2a phenotype. However, some researchers add an extra stimulation step using IL-13 or Toll-like receptor ligands.
3. The M2a phenotype may differ in morphology from conventional M2 macrophages, and the specific manifestations may vary depending on experimental conditions and stimulating factors.

Experimental Protocol for polarizing M0 macrophages into M2b macrophages.

1. Immune complexes (ICs) were prepared by mixing 15 μg/mL ovalbumin (OVA) and 150 μg/mL anti-OVA IgG for 30 min at room temperature (see Note 4).
2. The M0 macrophages were stimulated with LPS (100 ng/mL) and ICs for 24 h to obtain the M2b phenotype. The ICs doses, which are usually small, can be adjusted appropriately according to the actual situation.

Operation guide for polarizing M0 macrophages into M2c macrophages.

1. The M0 macrophages were stimulated with 10 ng/mL recombinant human interleukin-10 (IL-10) for 24–72 h to induce the M2c phenotype.

2. In the event that the induction is not fully achieved, the incubation period may be prolonged as deemed necessary.

Key points of steps for polarizing M0 macrophages into M2d macrophages.

1. The M0 macrophages were stimulated with 5 μmol/L 5'-*N*-ethylcarboxamidoadenosine (NECA) and 100 ng/mL LPS for 12–24 h to induce the M2d phenotype.

2. This step is optional. Recombinant human interleukin-6 (IL-6) can also be used to induce M2d macrophages, although the optimal concentration and incubation time still need to be determined.

As notes, the following points are crucial for the macrophage induction.

1. Employing a concentration of PMA lower than 10 ng/mL will result in inadequate differentiation. Conversely, a concentration of PMA higher than 100 ng/mL will have certain toxic impacts on cells. Considering that the length of the induction time is also critical for the differentiation effect of THP-1 cells, we propose that the optimal induction duration ranges from 24 to 48 h. Premature termination of induction, specifically when it is less than 24 hours, will lead to incomplete differentiation of THP - 1 cells. Conversely, if the induction period extends beyond 48 hours, the PMA concentration will diminish over time, causing the adherent cells to resume a rounded morphology and progressively detach from the culture vessel wall (Liu et al., 2023).

2. M0 macrophages were washed three times with prewarmed phosphate-buffered saline (PBS) to remove PMA and then replenished with cell culture medium. This step should be carried out quickly to prevent excessive water loss in cells. It is worth noting that whether to remove PMA during the subsequent induction is controversial. Removing PMA may cause adherent cells to become round again and gradually detach from the wall (see Note 1). However, in our experience, such outcomes do not always occur. Therefore, we recommend removing PMA to avoid influencing subsequent experiments.

3. For all experiments, M0 (unpolarized) macrophages were used as a control to confirm different macrophage polarizations.

4. Prepare the ICs IgG-OVA medium. IgG-OVA is produced by mixing anti-OVA IgG in a tenfold molar excess with OVA. Dispense 1 mL of complete medium into a 1.5 mL tube. Then, slowly add OVA and anti-OVA IgG (the proportion can be adjusted appropriately to facilitate the formation of the complex). Finally, rotate the mixture on a mini-rotator for 30 minutes at room temperature.

5. When the growth density is extremely low and the intercellular distance excessive, it results in cells secreting an insufficient quantity of secretory substances, consequently affecting intercellular communication. However, if the cell density is overly high, there will be insufficient growth space. The pseudopodia cannot be elongated or enlarged, leading to an unsatisfactory differentiation effect. Therefore, it is of particular importance to explore a growth density that is suitable for the differentiation of THP-1 macrophages.

Finally, we will recommend some potential markers. In fact, a number of potential markers have been proposed in accordance with previous reports (Huang, et al., 2018a; Li, et al., 2021; Li, et al., 2022). It should be noted that some of these markers have only been verified in THP-1 macrophages (Figure 1). According to our experience, it is relatively difficult to measure the expression of the cluster of differentiation 163 (CD163) and cluster of differentiation 206 (CD206) through flow cytometry experiments. This might be associated with differences in experimental methods or low levels of protein expression. Consequently, we suggest using RT-PCR (or qRT-PCR) first to identify macrophage markers. However, another study proposed that there could be disparities between gene expression and protein synthesis or that protein expression was sensitive to the biophysical alterations in the cellular microenvironment (Sapudom et al., 2020). These factors make it possible

that these two markers may be unstable. Additionally, although several studies have emphasized valuable markers, they had limitations. Therefore, considering the complexity of macrophage polarization, it is important to describe macrophage phenotypes using multiple markers.

Data availability statement

The data are available from the first author (pengfeilibio@163.com) on reasonable request.

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Author contributions

Experimental procedures were given in detail by Pengfei LI and Lin CHEN. Suggestions for improvements were made by Pengfei LI and Xingqiang LI. Pengfei LI, Wei YUAN, and Xuesong FENG prepared and approved the final version of the manuscript.

Compliance with ethics guidelines

Pengfei LI, Lin CHEN, Wei YUAN, Xingqiang LI and Xuesong FENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

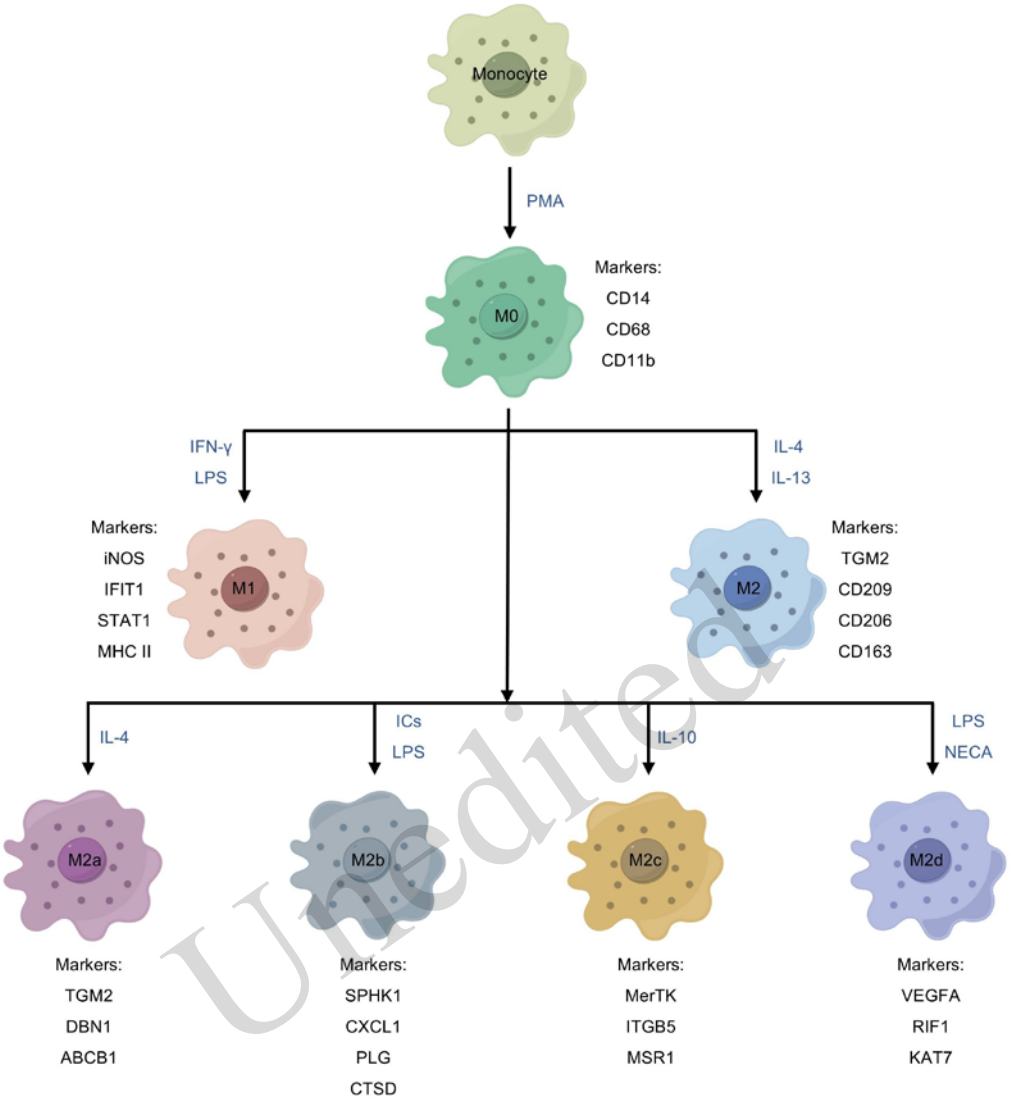

Figure 1. Potential markers in different macrophage subtypes. PMA: phorbol-12-myristate-13-acetate; IFN-γ: interferon-γ; LPS: lipopolysaccharide; IL-4: interleukin-4; IL-13: interleukin-13; IL-10: interleukin-10; ICs: immune complexes; NECA: 5'-*N*-ethylcarboxamidoadenosine; CD14: cluster of differentiation 14; CD68: cluster of differentiation 68; CD11b: CD11 antigen-like family member B; iNOS: inducible nitric oxide synthase; IFIT1: interferon-induced protein with tetratricopeptide repeats 1; STAT1: signal transducer and activator of transcription 1; MHC II: major histocompatibility complex class II; TGM2: transglutaminase 2; CD209: cluster of differentiation 209; CD163: cluster of differentiation 163; CD206: cluster of differentiation 206; DBN1: drebrin 1; ABCB1: adenosine triphosphate-binding cassette subfamily B member 1; SPHK1: sphingosine kinase 1; CXCL1: C-X-C motif chemokine ligand 1; PLG: plasminogen; CTSD: cathepsin D; MerTK: Mer receptor tyrosine kinase; ITGB5: integrin subunit β5; MSR1: macrophage scavenger receptor 1; VEGFA: vascular endothelial growth factor A; RIF1: replication timing regulatory factor 1; KAT7: lysine acetyltransferase 7.

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