Methodological benchmarking of human monocyte-derived macrophages in 2D and 3D collagen I culture

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Abstract

Macrophages are key regulators of tissue repair and wound healing. However, their reliable contribution to in vitro models remains challenging due to experimental variability. This study establishes a standardized framework for benchmarking human monocyte-derived macrophages under defined conditions. The effects of monocyte cryopreservation, cytokine-driven polarization, and detachment methods were systematically evaluated in 2D culture. Phenotypes were assessed by gene and protein markers, cytokine secretion, and phagocytic activity. While core macrophage identity and function were preserved, polarization markers and cytokine profiles varied depending on the protocol, with optimized conditions yielding more consistent subtype signatures. Importantly, macrophage viability and phenotypic stability were maintained in 3D collagen-based cultures, demonstrating the transferability of this approach.

Methods

Human Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats by density gradient centrifugation, followed by CD14+ monocyte selection using magnetic sorting. Monocytes were either used fresh or cryopreserved prior to differentiation into macrophages (M0) using M-CSF (Figure 1).

Macrophage polarization was induced using two protocols: an "old" and an optimized "new" condition. M1 macrophages were generated using LPS (1 ng/mL) alone or LPS combined with IFN-γ (20 ng/mL), while M2 macrophages were stimulated with IL-4 (20 ng/mL) alone or IL-4 in combination with IL-13 (20 ng/mL), respectively.

Phenotypic characterization included gene expression analysis (RT-qPCR), surface marker profiling (flow cytometry), and immunofluorescence imaging.

For 3D validation, polarized macrophages were embedded in collagen type I hydrogels, including fibroblast co-cultures, and assessed for gene expression.

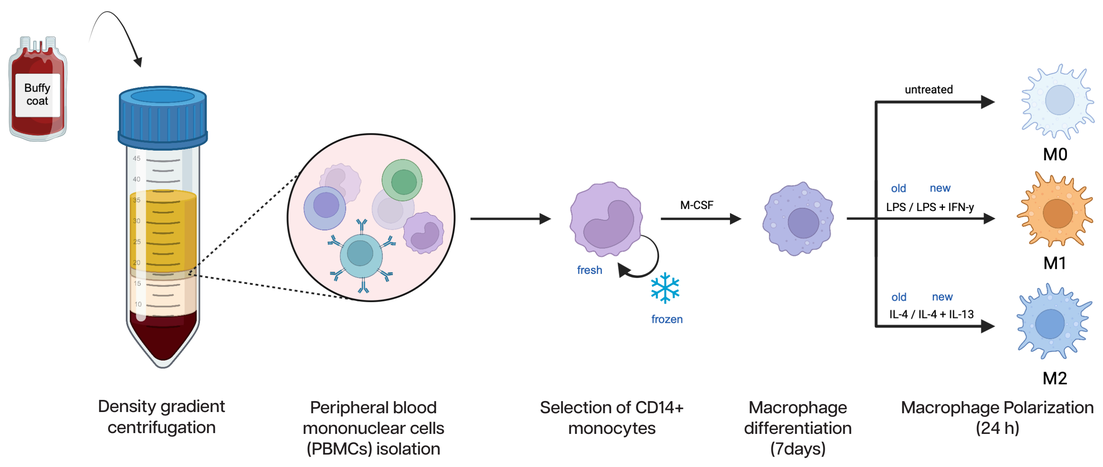


Figure 1: Workflow for human monocyte-derived macrophage differentiation and polarization. Peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats by density gradient centrifugation, followed by CD14+ monocyte selection. Monocytes were used either fresh or after cryopreservation and differentiated into macrophages (M0) using M-CSF over 7 days. Polarization was induced for 24 h using two protocols: M1 macrophages were generated with LPS alone (old) or LPS + IFN-γ (new), while M2 macrophages were stimulated with IL-4 alone (old) or IL-4 + IL-13 (new).

Results

Human monocyte-derived macrophages maintained stable expression of pan-macrophage marker (CD68, CD11c) across all conditions, indicating robust differentiation (Figure 2A, 3A). In contrast, polarization-specific markers were strongly dependent on cytokine stimulation, with combined protocols (LPS + IFN-γ; IL-4 + IL-13) yielding more distinct subtype signatures (Figure 2B-C, 3B-C, 4). Cryopreservation and detachment methods introduced moderate variability in marker expression but did not affect overall macrophage identity or viability.

Importantly, macrophages remained viable and phenotypically stable in collagen-based 3D cultures, including fibroblast co-cultures, supporting the transferability of this approach to physiologically relevant models (Figure 5).

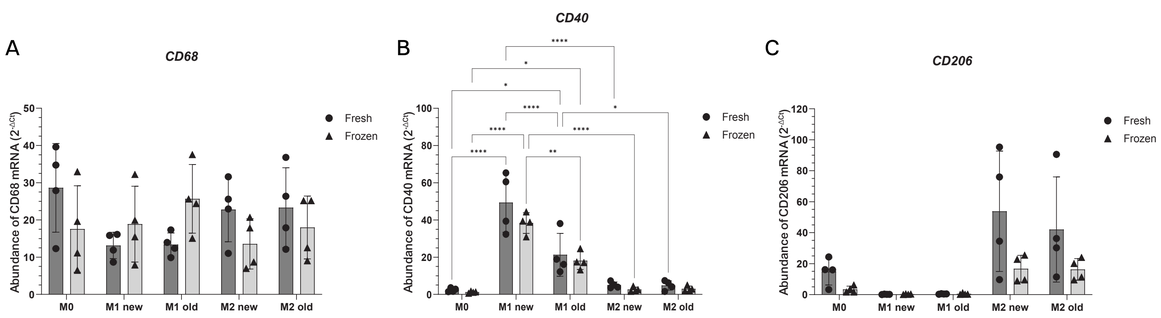


Figure 2: mRNA abundance of pan-macrophage and M1 and M2 phenotypic markers. (A) CD68, **(B)** CD40, **(C)** CD206 mRNA levels are shown as fold changes relative to the housekeeping gene GAPDH and normalized to a calibrator comprising all donors and cell types pooled. Macrophages derived from fresh monocytes are represented by circles, while those derived from frozen monocytes are indicated by triangles. Data are shown as bars of mean ± SD, n=4 biological replicates per condition. Mixed-effects analysis followed by Tukey's multiple comparison tests was used to calculate p-values; P > 0.05; *P ≤ 0.05, ** ≤ P0.01, ***P ≤ 0.001, ****P ≤ 0.0001.



Figure 3: Surface expression of pan-macrophage and M1 and M2 phenotypic markers as assessed by flow cytometry. Frequency of Parent [%] of **(A)** CD11c, **(B)** CD40, **(C)** CD200R is shown for macrophages differentiated using either the new or old protocol, from fresh (circles) or frozen (triangles) monocytes. Data are shown as bars of mean ± SD, n=3-4 biological replicates per condition. Mixed-effects analysis followed by Tukey's multiple comparison tests was used to calculate p-values; P > 0.05; *P ≤ 0.05, ** ≤ P0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

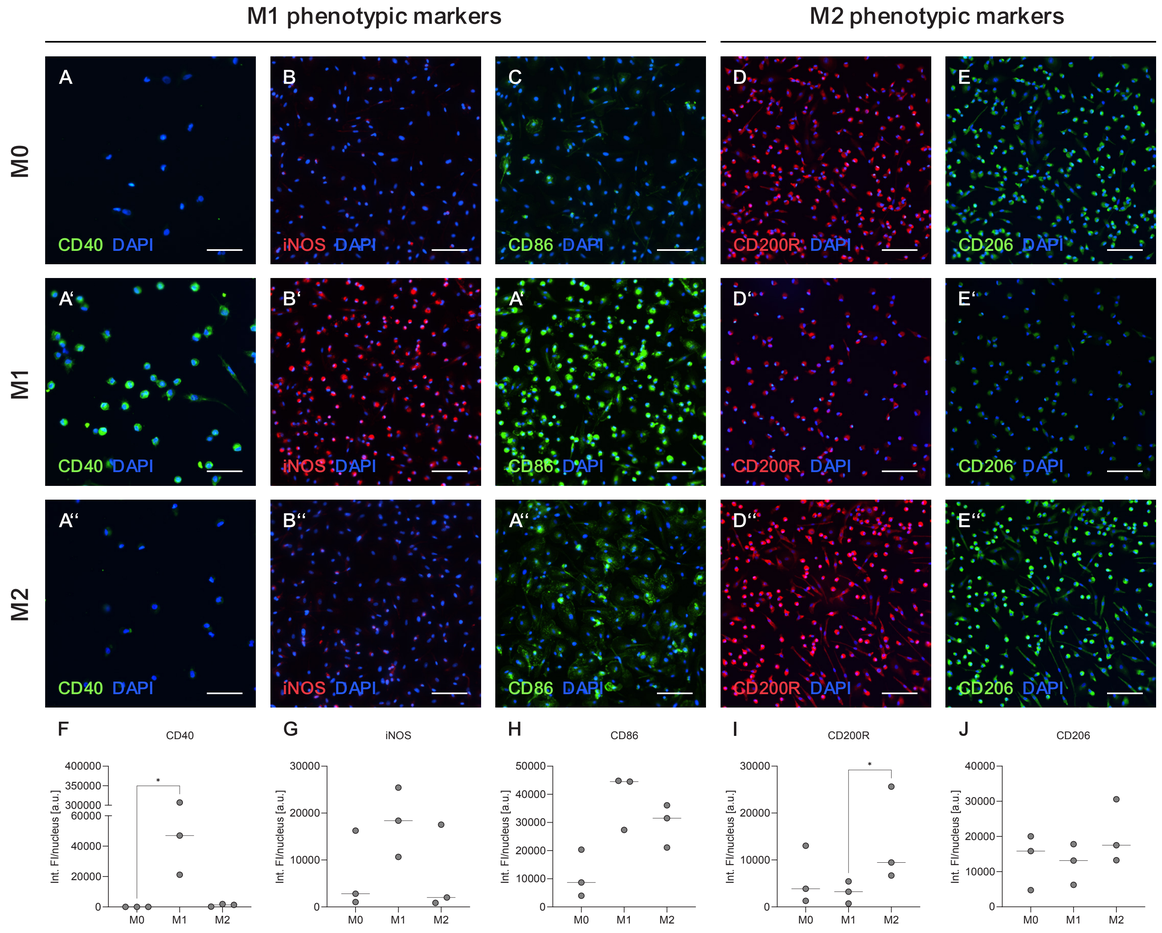


Figure 4: Immunofluorescence analysis of M1 and M2 phenotypic surface markers in human macrophage subtypes. Macrophages were isolated from peripheral blood of healthy donors (n = 3); three random fields per donor were acquired. **(A-A"–E-E")** Representative images of macrophages stained for CD40 (green), iNOS (red), CD86 (green), CD200R (red), and CD206 (green), respectively. Nuclei were stained with Hoechst (blue). **(F–J)** Quantification of integrated fluorescence intensity per nucleus [a.u.] for each marker, normalized to nuclear count (Int. FI/nucleus [a.u.]). Statistical significance was assessed using the Friedman test with Dunn's post hoc multiple comparisons. *P ≤ 0.05. Scale bars: 50 µm.

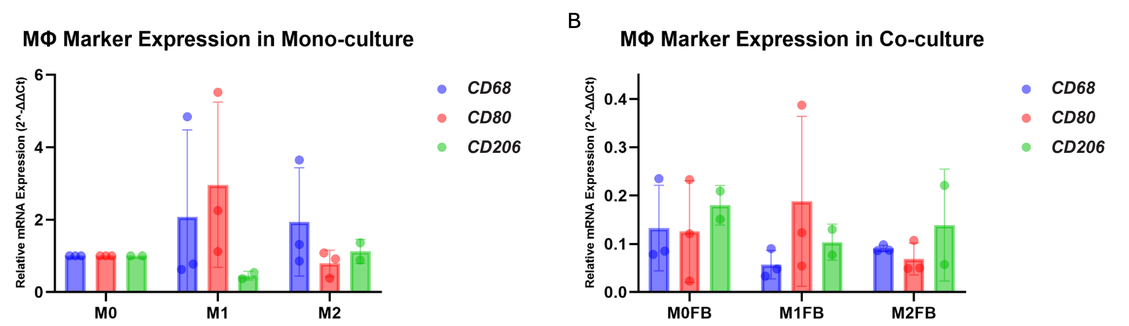


Figure 5: Gene Expression Analysis in 3D Collagen Hydrogels. Relative mRNA expression of the pan-macrophage marker CD68 and polarization markers CD80 (M1) and CD206 (M2) was quantified in collagen type I hydrogels after 3 days in culture. Macrophages were analyzed under **(A)** monoculture and **(B)** fibroblast co-culture conditions across M0, M1, and M2 states. Gene expression levels were calculated using the ΔΔCt method, normalized to GAPDH, and expressed relative to M0 monoculture controls. Data represent n = 2–3 biological replicates per condition. Statistical significance was determined by two-way ANOVA.*P ≤ 0.05.

Conclusion

Macrophages retained a stable core identity across conditions, while polarization-specific markers were highly dependent on cytokine stimulation and experimental setup. In contrast, functional properties such as phagocytosis remained robust. These findings highlight the importance of standardized protocols for reproducible macrophage characterization. Importantly, the approach was successfully translated to 3D collagen-based cultures, supporting its relevance for physiologically relevant models.

Future work should focus on standardization across laboratories and validation in more complex 3D and co-culture systems to better capture in vivo-like macrophage behavior.

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